On the origins and properties of *Verticillium* isolates associated with Crucifers

Thesis

ON THE ORIGINS AND PROPERTIES OF *VERTICILLIUM* ISOLATES ASSOCIATED WITH CRUCIFERS

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ABSTRACT

It had been proposed that long-spored isolates of *Verticillium* from crucifers were amphihaploid interspecific hybrids between *V. dahliae* and *V. albo-atrum*. The original aims of this thesis were to construct artificial hybrids between these two species and analyse the hybrids through molecular, genetic and pathological means. It was shown that limited recombination is possible between *nit* mutants of each species. Through pathogenicity testing on oilseed rape it was shown that long-spored amphihaploid isolates could be differentiated from haploid *V. dahliae* isolates. However, during this work it was found that of the non-*V. dahliae*-like supposed 'parent' was unlike any isolate of *V. albo-atrum* found to this day, and this species was unlikely to be involved in the hybridisation events that produced amphihaploid isolates of *Verticillium*. The projects main aims were revised to further characterise the long-spored isolates molecularly and through pathogenicity testing so their 'parental' origins were better understood. Molecular analysis of the rRNA gene V-region and ITS showed that a disease outbreak in cauliflowers in Belgium was caused by isolates molecularly similar to those from oilseed rape in Europe, and that isolates from Lucerne in Iran were *V. albo-atrum*. This thesis reports the first direct evidence that long-spored amphihaploid isolates of *Verticillium* from crucifers are interspecific hybrids through amplification of the 5*S* rRNA IGR and the β-tubulin gene. Sequence analysis of these along with the mitochondrial cytochrome B gene proved that *V. albo-atrum* was not a 'parent' of the amphihaploid isolates, but the 5*S* rRNA IGR supports the further division of *Verticillium* amphihaploids AFLP group β, to form a third group γ, represented by isolates from horseradish in Illinois, USA. Although the 'unknown parent' involved in the hybridisation events has yet to be found, through the results from this thesis it would now be possible to identify it molecularly.

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ABBREVIATIONS

AFLP	-	Amplified fragment length polymorphism
BM	-	Basal medium
bp	-	base pair
DNA	-	Deoxyribonucleic acid
d.p.i	-	Days post inoculation
EDTA	-	Ethylene diamine tetra-acetic acid
IGR	-	Intergenic region
IGS	-	Intergenic spacer
ITS	-	Internal transcribed spacer
kb	-	Kilobase pair
L	-	Lucerne
LB	-	Luria-Bertani medium
Mb	-	Megabase pair
MM	-	Minimal medium
mtDNA	-	Mitochondrial DNA
Nit	-	Nitrate-nonutilising
NL	-	Non-Lucerne
PCR	-	Polymerase chain reaction
PLYA	-	Prune lactose yeast extract medium
psi	-	Pounds per square inch
RAPD	-	Random amplified fragment length polymorphism
RE	-	Restriction endonuclease
RFLP	-	Restriction fragment length polymorphism
RO	-	Reverse osmosis
rRNA	-	Ribosomal RNA
SDW	-	Sterile distilled water
VCG	-	Vegetative compatibility group

1 GENERAL INTRODUCTION

1.1 Basic Taxonomy of *Verticillium*

1.1.1 The genus *Verticillium*

Nees von Esenbeck erected the genus *Verticillium* in 1816 (Isaac, 1967). A typical and classifying feature of this genus is that during asexual reproduction, verticilliate conidiophores develop bearing several phialides arising in groups at intervals along the conidiophore. At the apex of each phialide, a spherical head is formed consisting of large numbers of conidia held in watery mucilage.

Within the genus, diverse clades are formed comprising saprophytes and parasites of higher plants, insects, nematodes, mollusc eggs and other fungi (Heale, 1988) thus it can be seen that the genus is a wide ranging group of taxa characterised by simple but ill-defined characters. The genus may be broadly divided into three ecologically based groups i) mycopathogens, ii) entomopathogens (which are somewhat related to the mycopathogens) and iii) plant pathogens and related saprophytes (Carder and Barbara, 1991; Jun *et al.*, 1991; Barbara and Clewes, 2003).

Recently, the genus *Verticillium* underwent revision within the section *Prostrata*, which accounts for the majority of entomogenous and mycologolous species (Zare and Gams, 2001). This revision places the majority of species in the genus *Lecanicillium* and these form two groups; those that are like *V. lecanii* and those that are like *V. psalliotae*. However, this revision of *Prostrata* to *Lecanicillium* is not straightforward, as there are

isolates similar to *V. lecanii* within the *Prostrata* that do not clearly belong to *Lecanicillium* and form a distinct clade, for which the new genus *Simplicillum* has been introduced. Other species that are mainly parasites of nematode cysts and eggs such as *V. chlamydosporium* and *V. suchlasporium* are placed into *Pochonia*. The genus *Haptocillium* has been utilised for those nematophagous species that have adhesive conidia (Zare and Gams, 2001).

1.1.2 *Verticillium* section *Nigrescentia*

For my studies the plant pathogenic *Verticillium* shall be of sole concern. These species fall within the section *Nigrescentia* as described by Gams and van Zaayen (1982). The plant pathogenic *Verticillium* are, for the main part, classified on the basis of the type of melanised resting structures produced. The two better known plant pathogens, *V. dahliae* Klebahn (1913) and *V. albo-atrum* Reinke and Berthold (1879) produce microsclerotia and dark resting mycelium (Figure 1.1 a, b) respectively and this classic mode of differentiation, first reported in the late 1950s (Isaac, 1957) is still used as initial marker of species. A third species, *V. tricorpus* Isaac (1953) (Figure 1.1 d) produces microsclerotia, dark resting mycelium and chlamydospores. Although this species can cause disease in tomato and cotton, it tends to be less pathogenic than *V. dahliae* and *V. albo-atrum*. Other related plant pathogenic *Verticillium* species are *V. nubilum* Pethybridge (1919) and *V. nigrescens* Pethybridge (1919) (Figure 1.1 c, e). These species produce chlamydospores alone, but they are weak pathogens that verge on saprophytes in nature and the results of their infection in a host crop are of much less importance than those of *V. albo-atrum* and *V. dahliae*.

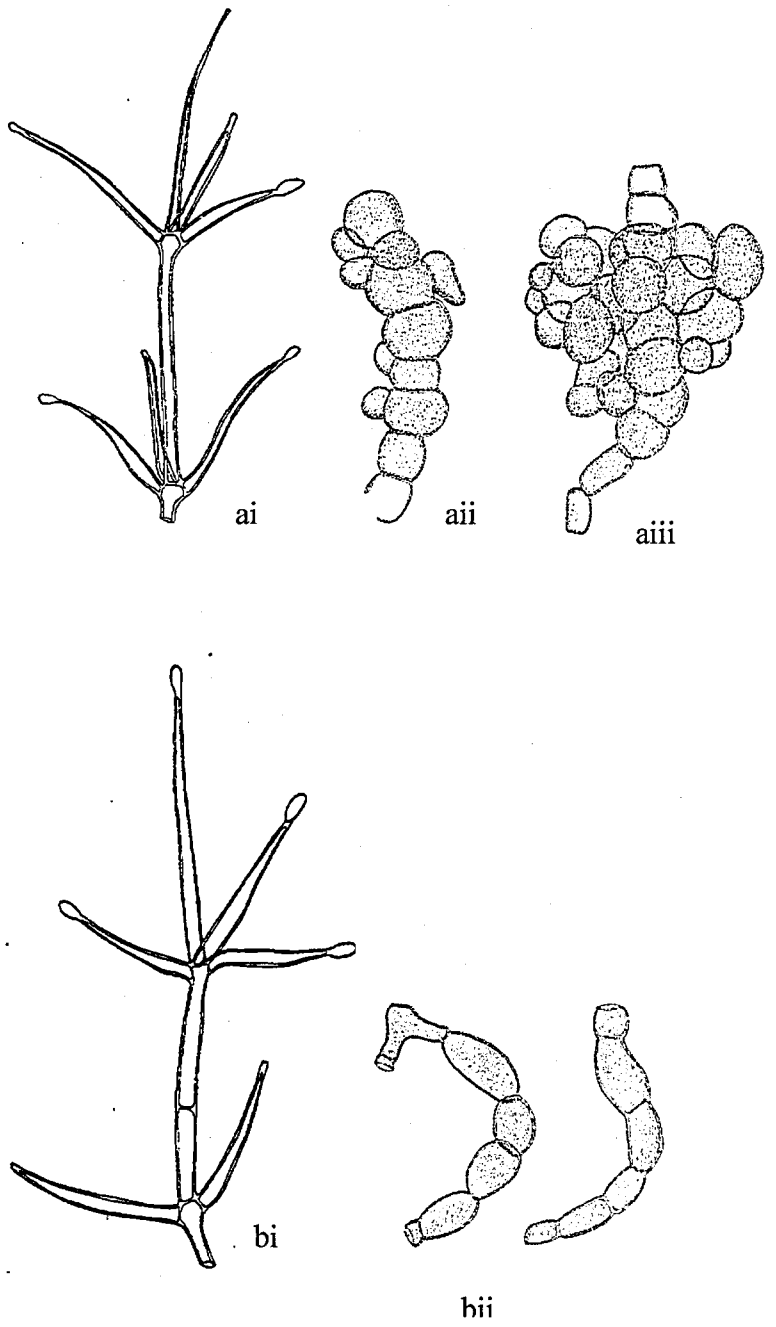

Figure1.1. Schematic drawings of the conidiophore and resting structure of plant pathogenic *Verticillium* species. ai., *V. dahliae* conidiophore; aii., *V. dahliae* immature microsclerotium; aiii., *V. dahliae* mature microsclerotium; bi., *V. albo-atrum* conidiophore; bii., *V. albo-atrum* dark resting mycelium. Taken from CMI descriptions of pathogenic fungi and bacteria 255-260. Continued.

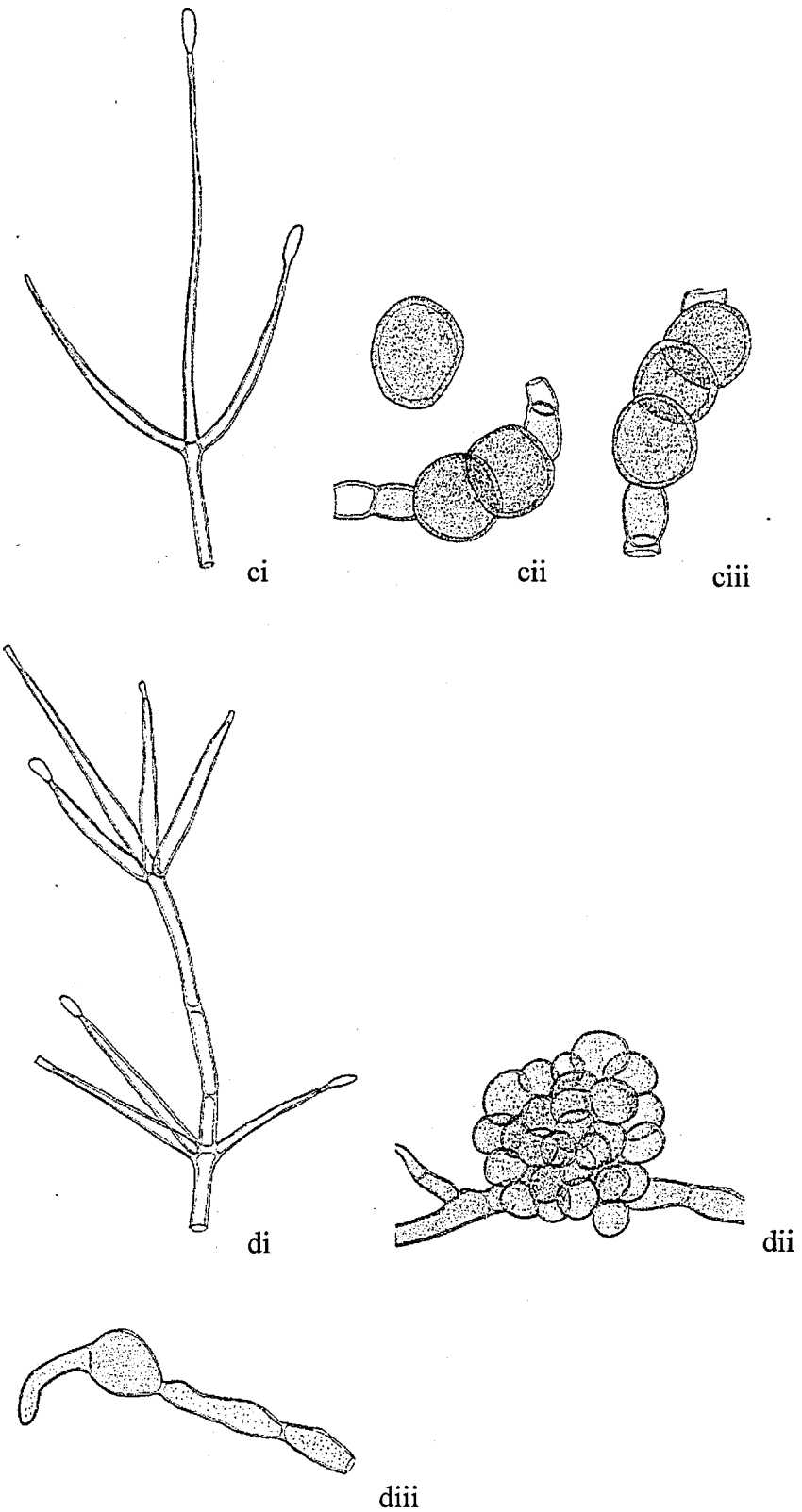

Figure 1.1 ci., *V. nubilum* conidiophore; cii., *V. nubilum* chlamydospore. di., *V. tricorpus* conidiophore., dii., *V. tricorpus* microsclerotium; diii., *V. tricorpus* chlamydospore. Taken from CMI descriptions of pathogenic fungi and bacteria 255-260. Continued.

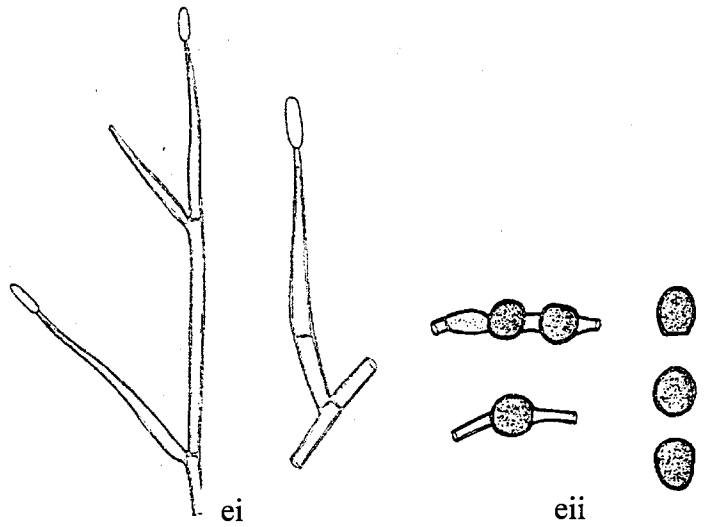

Figure 1.1. ei., *V. nigrescens* conidiophore; eii., *V. nigrescens chlamydospores.* Taken from CMI descriptions of pathogenic fungi and bacteria 255-260.

Where they infect plants, all these species are typically vascular wilt pathogens (see below). Another pathogen, *V. theobromae* (Turconi) Mason and Hughes (in Hughes 1951), is not a vascular pathogen and is associated more with fruit rots such as 'cigar end' of banana and produces olivaceous coloured hyphae on the appropriate medium (Pegg and Brady, 2002).

None of the above species have known teleomorphs and analysis of gene sequences indicates that they align with hyphal ascomycetes as anamorphic Phyllachorales (Messner *et al.*, 1996; Seifert and Gams, 2001). This study shall, for the purposes of clarity, describe isolates of plant pathogenic *Verticillium* by their given species name as determined by previous authors. This is, however, not to be deemed as fixed and the discussion will reflect the validity of each species and their names in the light of results presented and their implications on the plant pathogenic *Verticillium* species complex. Many of the issues specifically relating to classification of the plant pathogenic species have been reviewed

recently (Barbara and Clewes, 2003). The isolates studied are listed as the species as they were supplied us; should contradictory evidence be presented, this is discussed.

1.2 *Verticillium* wilt disease

1.2.1 Economic implications and disease control

The impacts of *V. dahliae* and *V. albo-atrum* infections are economically substantial, as they are responsible for losses due to wilt in over 300 dicotyledonous crops globally. These include important glasshouse crops such as tomato and chrysanthemum, the traditional brewing industry staple hops, and crops of national and world-wide significance like cotton. For example, *Verticillium* wilt of cocoa (*Theobroma cacao)* caused by *V. dahliae* is a serious problem in Brazil, which is the second largest producer of cocoa in the world. It was also linked to the failure of the cocoa industry to establish itself in Uganda (Cooper *et al.,* 1997).

Wilt diseases are also important in developed countries *e.g. Verticillium* wilt caused by *V. dahliae* and *V. albo-atrum* limits potato production within the USA. This problem is exacerbated by a synergistic interaction between these species with a root lesion nematode, *Pratylenchus penetrans*. The two fungal pathogens alone, or in conjunction with the latter nematode cause a disease known as potato early dying (PED). The potato is the most important vegetable crop in North America. In 2000, 690 000 hectare were grown, with an average yield of 32.6 metric tonnes per hectare and a farm gate value of more than $2.7 billion. PED is widespread in the USA thus, if wilt develops through a growing season, then severity increases during the period of maximum tuber bulking, a significant

reduction in tuber size and marketable yield can result. In North America, yield reduction can be as much as 10-15% in a moderately diseased field, and in highly affected fields 30-50%. The economic impact of PED across the potato industry is significant because of direct losses due to low yields and because expensive preplant soil fumigation has become a routine disease management practice (Rowe and Powelson, 2002). Hence, the potential socio-economic decline of the potato industry attributed to *Verticillium* infection may prove to be unremitting.

The difficulty in managing *Verticillium* wilt arises from three main factors; the ability of its resting structures to persist in the soil, its wide host range and that during its growth in the vascular tissue of the host crop the fungus is relatively inaccessible. Because of these factors control of *Verticillium* wilt is often said to be ideally mediated through resistant or tolerant cultivars. One example is tomato cultivars that carry the *Ve* resistance gene. But, it is apparent that some isolates of *V. albo-atrum* are able to overcome this mechanism of resistance (O'Neill and Barbara, 2002). Hop breeders, however, have managed to stay 'one step ahead' by producing varieties with effective levels of resistance, even in the face of increasingly aggressive fungal isolates, and are widely employed. For the majority of hosts, resistant varieties are unavailable and likely to remain so for the near future, hence many disease control strategies focus on reducing the number of infective propagules in the soil. Success has been achieved in Mediterranean countries using soil solarisation techniques, and steaming has been as successful. For many growers, however, the control method of choice remains the application of chemical treatments such as chloropicrin, metham-Na or methyl bromide.

Crop rotation is a cultural technique recommended for control of *Verticillium* wilt, though it rarely is able to eradicate the problem due to the broad host range of the fungus and the persistence of the resting structure in the soil. For this method to be effective, the crop chosen to rotate with the susceptible host must a) result in reduction of resting structure in the soil and parallel reduction of wilt in the susceptible crop; b) be compatible with current production practices and finally c) result in acceptance by the grower of crop rotation (Xiao *et al.*, 1998). Certain crucifer residues can result in disease suppressive effects, possibly related to the breakdown products of glucosinolates, sulphur containing compounds often characteristic of *Brassica* species (Subbarao and Hubbard, 1995).

1.2.2 Disease cycle

The fungus persists within the soil environment as melanised resting structures and germination is thought to be initiated from these by root exudates. Entry into plants may occur through direct penetration of the epidermal cells by the fungus or through wounds. As previously touched upon, in some hosts, particularly potatoes, root damaging nematodes will increase disease severity (Botseas and Rowe, 1994). The nematodes are not thought to act as vectors but to allow the fungus entry through root damage. The hyphae then grow biotrophically through the root cortex to the endodermis and finally invade the vascular system. Once in the vascular system, the pathogen progresses dimorphically through mycelial growth and the production of conidia. Rapid spread of the fungus is perpetuated throughout the plant via the transpiration system and the flow of the xylem is soon obstructed not only by conidia and mycelium but also by gels, tyloses and gums that are formed. (Figure 1.2)

The disruption in the transpiration stream results in symptoms of wilt; typically there is loss of turgour and chlorosis of shoots and leaves and host death normally follows (Hiemstra, 1998). The host plant is then subjected to a necrotophic stage in which plant tissues are lysed leading to the utilisation of the host's food reserves by the fungus. As direct consequence of necrotrophy, there is a substantial increase in fungal biomass and the production of resting structures. These resting structures remain with the decomposing host tissue and eventually pass on into the soil where they may remain in mycostasis for many years (Heale, 2000).

In some tree species, *Verticillium* wilt does not necessarily mean death for the plant, even when serious damage is suffered in the season of infection. The potential for recovery in some tree species results from the highly compartmented structure of the xylem and the presence of a cambium that is able to produce new layers of tissue around old, diseased xylem. Trees do not overcome injuries and infections by healing the affected tissues but by isolating the offending part and replacing it with new, healthy tissue (Hiemstra, 1998).

Distribution of the fungus is limited by its soil-borne nature; dispersal between fields tends to be through movement of infected plant material or infested soil. Movement of infected material can mean not only the human movement of whole plants, but also wind dispersal of plant debris, such as fallen leaves. Excess irrigation and dust storm may also disseminate the pathogen if the resting structures are located towards the surface of the soil (Hiemstra, 1998). The transmission of the pathogen by aphid vectors has been recorded (Huang *et al.*, 1983) allowing the dispersal of the pathogen between fields. However, whether this is an effective method of disease transmission must be queried as the underlying pathology of the disease dictates that infection occurs from the germination of

resting structures whose hyphae penetrate into the xylem. The main opportunities for far reaching dispersal of *Verticillium* arise through infected plant propagation material.

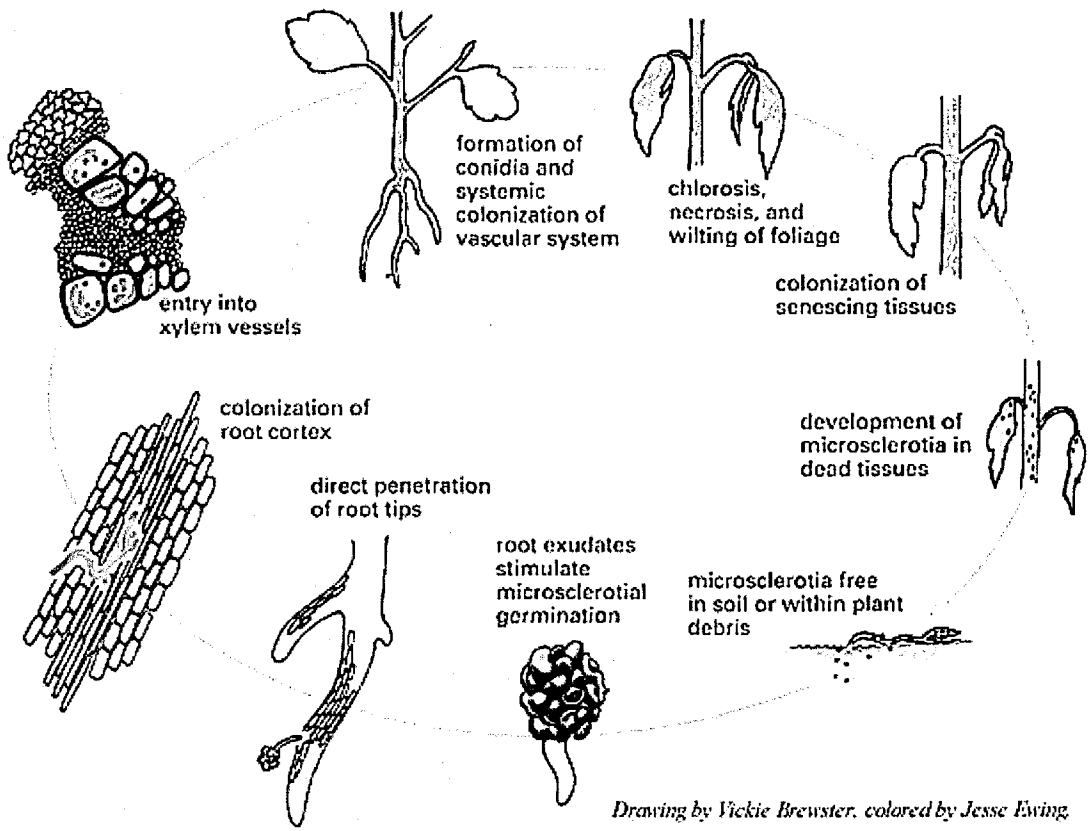

Figure 1.2. A schematic diagram representing the disease cycle of *V. dahliae*. http://www.bcc.orst.edu/bpp/bot350/. © Copyright 2000 by The American Phytopathological Society

1.3 Molecular differentiation of plant pathogenic *Verticillium*

1.3.1 Molecular analysis supports distinction between plant pathogenic *Verticillium* species

The species limits, defined firstly by morphological, biochemical and physiological identification, are well supported by molecular evidence for most isolates of the main plant pathogenic species of *Verticillium*.

Typas *et al.* (1992) used RFLP analysis of the mtDNA and rRNA gene repeat unit. RFLP analysis of the functional rRNA genes complex could not distinguish between *V. dahliae* and *V. albo-atrum*, but could distinguish between these and the other plant pathogenic species, *V. tricorpus*, *V. nigrescens* and *V. nubilum*, as well as some entomopathogenic species. The mtDNA probe, however, discriminated between *V. dahliae* and *V. albo-atrum* and also separated them clearly from the other species tested. Similar evidence was presented by Carder and Barbara (1991), who utilised random genomic probes to detect RFLPs to discriminate between mainly plant pathogenic species and an entomopathogen. Okoli *et al.* (1993, 1994) also discriminated between *V. dahliae* and *V. albo-atrum* using RFLP analysis of the genomic DNA. Further evidence supported this data through amplification and the sequence analysis of the rRNA gene internal transcribed spacer (ITS) regions (Morton *et al.*, 1995b) and intergenic sequences (IGS) (Morton *et al.*, 1995a).

1.3.2 Identification of sub-specific groups within *V. dahliae* and *V. albo-atrum* based on host-origin

Molecular techniques have divided *V. dahliae* and *V. albo-atrum* into sub-specific groups based on host origin. For example analysis of *V. albo*-atrum revealed that this species could be sub-divided according to virulence to a particular crop, Lucerne (*Medicago sativa*). These groups, termed Lucerne (L) and Non-Lucerne (NL), were essentially identified by RFLP (Carder and Barbara, 1991; Typas *et al.*, 1992). Later work corroborated these findings using randomly amplified polymorphic DNA (RAPDs) (Barsubiye *et al.*, 1995) and at a single locus, the rRNA gene repeat unit ITS region (Morton *et al.*, 1995b). Sequence analysis of the sub-repeats within the IGS by Collins *et al.* (2003) found a 320 bp imperfect repeat that was termed 'V-region', importantly this provided strong supporting evidence that Lucerne and Non-Lucerne isolates were distinct, separating the two groups of isolates by more 31 bp *cf.* 1 bp separation by ITS1 and ITS 2. The importance of V-region will be discussed later in the General Introduction and in Chapter 5.

Similar analyses of *V. dahliae* alluded to sub-specific groups that could be correlated with particular hosts. RFLP analysis of *V. dahliae* isolates mainly from the UK recognised two groups, defined as A and B. Isolates from peppermint (*Mentha* x *piperita*) formed a host-adapted sub-specific group (M) within *V. dahliae* that was distinct from the non-host adapted groups A and B of *V. dahliae* present in the UK (Okoli *et al.*, 1994). A few remaining UK isolates were unable to be placed in a designated RFLP group. Studies of *V. dahliae* isolates mainly from Japan that had been previously grouped according to their pathogenicity clustered similarly by RFLPs, except some isolates that would only infect

eggplant (*Solanum melongena*) and turnip (*Brassica campestris* spp. *rapifera*), the group C of Horiuchi *et al.* (1990). None of these RFLP groups corresponded with the UK groups (Carder and Barbara, 1994; Koike *et al.*, 1996). It largely seems that within *V. dahliae* world-wide there is continuous variation with the distinction of sub-groups reflecting specific geographical areas or the introduction of 'alien' isolates into a previously homogenous population.

1.3.3 A proposed 'new' species of *V. albo-atrum*

A further group of isolates classified as *V. albo-atrum* on the basis of production of dark resting mycelium-like resting structures, and referred to as Group II (Robb *et al.*, 1993), have been proven by molecular methods to be best regarded as a new species (Morton *et al.*, 1995a,b; Mahuku and Platt, 2002). These isolates (still referred to in the literature as group II) are more related to *V. tricorpus* than *V. albo-atrum* isolates of the Lucerne and Non-Lucerne pathotypes (together often referred to as group I). Group II isolates are also morphologically distinguished from group I isolates as it was observed that their dark resting mycelium occurs in bundles unlike that of group I isolates (Robb *et al.*, 1993).

1.4 *Verticillium* isolates from Crucifers

1.4.1 Occurrence of isolates in Crucifers

Most isolates of *V. dahliae* and *V. albo-atrum* are considered not to be pathogens of crucifers but some isolates of *Verticillium* as host-adapted pathogens of crucifers have been reported. These crucifer isolates were originally classified as *V. dahliae* on the basis of

producing microsclerotia and have recently become significant pathogens of economically important crops. Isaac (1957) first recorded an isolate of *Verticillium* from crucifers from Brussel sprout plants (*Brassica oleracea* ssp. *gemmifera*) with wilt near Evesham, UK. Stark (1961) who isolated a strain from wilted horseradish (*Armoracia rusticana*) in Hamburg, Germany, recorded that the isolate had conidia approximately twice as long as other *V. dahliae* isolates and referred to it as *V. dahliae* var. *longisporum* (Stark, 1961).

Isolates similar to *V. dahliae* var. *longisporum* were found to be the cause of serious losses in oilseed rape (*Brassica napus* ssp. *oleifera*) in Northern Europe, and are the foundation for the 'misnamed' species-*V. longisporum* (Karapapa *et al.*, 1997). *Verticillium* isolates obtained from cauliflower (*Brassica olereacea* ssp. *botrytis*) crops in California, USA, that have similar morphological characteristics to other *Verticillium* crucifer isolates that have also been described (Subbarao *et al.*, 1995) as have isolates from horseradish in Illinois, USA (Eastburn and Chang, 1994).

1.4.2 Morphological and genetic observations of *Verticillium* isolates from Crucifers

The majority of *V. dahliae* and *V. albo-atrum* isolates are haploid, but many crucifer isolates have been shown to possess almost twice the DNA content of the haploid isolates and were assumed to be diploid. It was Ingram (1968), who unsuccessfully attempted to induce auxotrophic mutants in the *Verticillium* crucifer isolates utilising UV radiation, who initially investigated this supposition. Subsequent efforts by other workers to induce auxotrophic mutants in *Verticillium* crucifer isolates have also proven fruitless (Clarkson and Heale, 1985a, b; Hastie, 1973). Concomitantly, attempts to obtain microsclerotial colour mutants from *Verticillium* crucifer isolates from sugar beet and oilseed rape

(Puhalla and Hummel, 1983) after treatment by UV also proved unsuccessful. Other authors have failed to obtain nitrate-reductase mutants (Subbarao *et al.*, 1995) and melanin-deficient mutants (Nagao *et al.*, 1994) from *Verticillium* crucifer isolates. Conidial size and DNA content, as measured by Feulgen microdensitometry for *Verticillium* crucifer isolates are both approximately double that of other *V. dahliae* and *V. albo-atrum* (Karapapa *et al.*, 1997; Typas and Heale, 1977; 1980). More precisely, Karapapa *et al.* (1997) showed that crucifer isolates had *ca.* 1.78 times the amount of DNA of *V. dahliae*.

Enzymatic, morphological and pathological analyses of Japanese *V. dahliae* crucifer and non-crucifer isolates differentiated them into four distinct groups (Horiuchi *et al.*, 1990). Group A (eggplant strain) were of isolates pathogenic to eggplant and turnip, group B were pathogenic to tomato and turnip (tomato strain), group C were pathogenic to eggplant, sweet pepper and turnip (sweet pepper strain) and group D was pathogenic to turnip alone (crucifer strain). It was concluded by Horiuchi *et al.* (1990) that, based on host-specific pathogenicity testing, isolates of group D were solely obtained from cruciferous plants, whereas isolates of groups A, B, and C were from plants of various families including Cruciferae. In the text, the term *Verticillium* crucifer isolates refers to isolates that are pathogenic to crucifers alone.

1.4.3 Molecular differentiation of *Verticillium* crucifer isolates from other *V. dahliae* and *V. albo-atrum* isolates

As mentioned previously, molecular techniques have been utilised to discriminate intraspecifically some isolates of *V. dahliae* and *V. albo-atrum* parallel to host preferance. The relationships of *Verticillium* crucifer isolates to *V. dahliae* and *V. albo-atrum* have also

been considered in such a manner. RFLP analysis of a limited number of *Verticillium* crucifer isolates from Europe and Japan was undertaken by Okoli *et al.* (1994). This demonstrated that a group, D, separate from other *V. dahliae* and *V. albo-atrum* groups was formed. RFLP analysis of Japanese crucifer isolates with isolates of *V. dahliae* corroborated the data of Horiuchi *et al.* (1990), and found that this group corresponded with the Group D of Okoli *et al.* (1994) (Carder and Barbara, 1994).

Sequence analysis of the ITS of the rRNA genes of *Verticillium* crucifer isolates regions showed that they differed from *V. dahliae* isolates by at least 6 bp yet only differed by 1 bp from Lucerne isolates of *V. albo-atrum* (Morton *et al.*, 1995b). Further investigation by the same group found distinct variations in the rRNA gene intergenic regions (IGS) of *V. albo-atrum*, *V. dahliae* and *Verticillium* crucifer isolates thus supporting a proposition that all three are distinct species (Morton *et al.*, 1995a). RAPD studies verified the former findings in that the majority of *Verticillium* crucifer isolates form a distinct group from other *V. dahliae* isolates (Karapapa *et al.*, 1997; Koike *et al.*, 1996; Messner *et al.*, 1996).

1.4.4 Foundation for a new species?

Firstly, Karapapa *et al.* (1997) demonstrated a correlation between conidial length with nuclear diameter (r=0.856) illustrating the relationship that isolates with long spores have larger nuclear diameters than those with short spores. Similarly, these authors showed that there was a good correlation between conidial length and DNA content (r=0.908). However, four isolates designated as 'recombinants' (although the meaning of this term is not clear in this context) are excluded from the latter analyses. Significantly, of the isolates excluded are the original *V. dahliae* var. *longisporum* isolate (195) (Stark, 1961) and the Brussel sprout isolate (111) taken from Evesham, UK (Isaac, 1957) - the first recorded incidence of *Verticillium* wilt in crucifers. Karapapa *et al.* (1997) showed that isolate 111, through measurements of conidia and nuclear DNA content, although short-spored maintained high DNA content when compared to other short-spored isolates. Whereas isolate G19, an oilseed rape isolate, although long spored possessed a relatively low DNA content when compared to other long spored isolates. Thus, as mentioned previously, studies of conidial length with nuclear DNA content in the study of Karapapa *et al.* (1997) show good correlation for the isolates tested however, this analysis was flawed through the dismissal of some reputed short-spored crucifer isolates regarded as 'recombinant' and the over emphasis on isolates from a single host and geographical location, in effect giving a graph with only two points.

Other reportedly short-spored high DNA content *Verticillium* isolates (90-01, 90-02) taken from cauliflower (*Brassica oleracea.* var. *botrytis*) (Subbarao *et al.,* 1995) in California were reported two years previously to the publication of Karapapa *et al.* (1997) but were not included in the latter paper. However, these isolates from cauliflower crops were later

shown to be long spored, high DNA content like oilseed rape isolates (Collins *et al.*, 2003). Measurements of isolate 111 have not been undertaken by any authors other than Karapapa *et al.* (1997), and until this is repeated, or other putative short-spored high DNA content spores are isolated and analysed in the same manner, the status of isolate 111 must be questioned (after so many years the culture used by Karapapa *et al.* (1997) may not, of course, still be the same isolate).

1.4.5 Terminology

Karapapa *et al.* (1997) proposed that the term 'near-diploid' be used to indicate the disproportionate DNA content of *Verticillium* crucifer isolates. The term 'near-diploid' has no precedence in literature nor does it indicate any functional genetic structure of *Verticillium* crucifer isolates. Classically, the term diploid is a genetic one and refers to having paired homologous chromosomes so that twice the haploid number is present. However, Karapapa *et al.* (1997) propose that that *Verticillium* crucifer isolates arose through the hybridisation of distinct species and recombination between the two genomes. Thus, the resultant genetic state is not diploid and the term 'near-diploid' does not indicate the hybrid origin or genomic arrangement of *Verticillium* crucifer isolates, but suggests that diploidy may have occurred at some point. At this time, all known crucifer isolates produce microsclerotia and are thus classified as *V. dahliae* var. *longisporum* as proposed by Stark (1961). The majority of these are long-spored and it was proposed by Karapapa *et al.* (1997) that some of these isolates should form the foundation for a new species termed *V. longisporum*; this new name has been used by some authors but has not yet been formally accepted. The assembly of a new species is somewhat hasty and under-estimates the intricacy of the situation. Additionally, as previously touched upon, the use of

ambiguous terms or unexplained terms such as 'near-diploid' and 'recombinant' does not help to clarify the situation. By comparison, the term 'amphidiploid' is used in some plant species to indicate the combination of two diploid species. Thus a more accurate description of the genetic re-assortment undergone in *Verticillium* crucifer isolates is 'amphihaploid' (Collins *et al.*, 2003) ('allodiploid' is a term with similar meaning and precedent in the accepted term 'allotetraploid' used to describe plants). In the text when referring to the chromosome complement and genetic state of long-spored crucifer isolates the term 'amphihaploid' will be employed.

Preliminary evidence to substantiate the proposed amphihaploid state of *Verticillium* crucifer isolates was provided through the inability to induce auxotrophic mutants in *Verticillium* crucifer isolates by other workers as mentioned previously. This was further supported by Karapapa *et al.* (1997) through the attempted production of haploid segregants from *Verticillium* crucifer isolates utilising ρ-fluorophenylalanine. From these latter results, Karapapa *et al.* (1997) concluded that the original assumption that crucifer isolates were diploid (Hastie, 1970) was incorrect. Karapapa *et al.* (1997) proposed that *Verticillium* crucifer isolates arose through a heterokaryon stage between two distinct parental species followed by nuclear fusion leading to a nucleus containing two distinct sets of chromosomes that, over time, through chromosomal re-arrangements produced an amphihaploid hybrid. Such an existence would continue, it is proposed, as lethal effects would be balanced in the hybrid whereas any potential haploid progeny might only survive through further chromosomal rearrangements. A parasexual cycle, as an alternative to conventional sexual recombination mechanisms, has been described for plant pathogenic *Verticillium* species in laboratory studies (Clarkson *et al.*, 1985a, b; Heale, 1988) and the conclusions presented by Karapapa *et al.* (1997) may be considered further evidence in

support of such a system operating in wild populations of filamentous fungi allowing for the infection of new hosts through generation of genetically distinct progeny. However, it could also be argued that amphihaploids suggest that between species the parasexual cycle does not occur as it normally aborts halfway with no reductive stage. The implications of parasexuality shall be discussed further in Chapter 3.

It was noted by Karapapa *et al.* (1997) that some crucifer isolates in their RAPD analysis shared distinct banding patterns with the Lucerne isolates of *V. albo-atrum* and not with any other Non-Lucerne isolates of *V. albo-atrum* tested in their study. Also, within the RAPD analysis, numerical taxonomy defined that *V. dahliae* as being 42% similar to the crucifer isolates tested. Thus, it was proposed that crucifer isolates of *Verticillium* arose through the hybridisation of a *V. albo-atrum* Lucerne isolate and a *V. dahliae* isolate. Karapapa *et al.* (1997) concluded that crucifer isolates of *Verticillium* should be assigned a new species name of *V. longisporum*. The authors defined that the species was distinct from *V. dahliae* by primarily three morphological characters that are common to most *Verticillium* crucifer isolates. These are as follows: 1. microsclerotia are elongate; 2. conidia are comparatively longer (7.1-8.8 μm) than that of *V. dahliae* (3.5-5.5 μm); 3. there are usually three phialides per conidiophore whereas *V. dahliae* has four to five phialides per node. It was further characterised by a lack of the extracellular polyphenol oxidase activity (apart from three isolates that were designated as 'recombinant' by the authors, that were all from crucifers and two of which were long-spored) that is common in the majority of *V. dahliae* and by having nuclear diameters of *ca.* 1.85 μm whereas the nuclear diameters of *V. dahliae* are *ca.* 1.16 μm (Karapapa *et al.*, 1997). The seemingly arbitrary exclusion of 'recombinant' isolates by Karapapa *et al.* (1997) coupled with the pronounced focus on isolates from oilseed rape may call into question any conclusions reached with

regard to the distinction of a new species encompassing just some long-spored isolates. Some of the difficulties associated with the decision to erect a new species based on long-spored isolates have been raised in Collins *et al.* (2003) and fully discussed in Barbara and Clewes (2003) who wrote "At best, only partial answers are currently available to several questions relating to amphihaploid isolates *viz.* (i) how many discrete groups exist, (ii) what are the identities of their parents, (iii) what maintains the stability of two discrete haploid genomes in single nuclei, and (iv) can definitive proof of their hybrid status be found. The status and origin of short-spored crucifer isolates are similarly unclear."

There is a more general issue in naming fungal hybrid isolates summarised by Brasier *et al.* (1999) "which as yet to be tested in mycology, is how to taxonomically define a hybrid or a hybrid complex in a way that is of practical use in quarantine legislation and diagnosis" leading Collins *et al.* (2003) to suggest the retention of *V. dahliae* for all isolates producing only microsclerotia. As work reported later in this thesis increases the apparent complexity grouping crucifer isolates, this suggestion will be adapted here.

1.5 Other plant pathogenic interspecific hybrids

1.5.1 Oomycetes

In the early 1990s, a previously undescribed *Phytophthora* that caused the death of riparian alders (*Alnus* sp.) was reported and characterised from Britain (Brasier *et al.*, 1995). This pathogen was found to occur across northern Europe, it is symptomatically and morphologically similar to *P. cambivora* (a non-pathogen of alder), but it differs from *P. cambivora* in that it is self-fertile, has a submerged instead of an aerial colony type, lower temperatures for growth and a high level of zygotic abortion (Brasier *et al.*, 1995; Brasier *et al.*, 1999).

In a seminal paper, Brasier *et al.* (1999) presented evidence that these *Phytophthora* alder pathogens were heteroploid interspecific hybrids involving a species-like *P. cambivora* and an unknown taxa possibly similar to *P. fragariae*. This was concluded through molecular evidence provided by AFLP and rRNA gene ITS sequence analysis, in addition to cytological evidence. The diploid chromosome number of *P. cambivora* was confirmed as n=10-12, *P. fragariae* was similarly shown also to be n=10-12, but the chromosome number for the alder pathogen was shown to be n=18-22 approximately tetraploid (some would say 'near-tetraploid') and in these chromosomal pairing was frequently seen but no secondary division stages observed. Such anomalies affirmed that the alder pathogen was a polyploid hybrid that, due to inequalities in chromosomal pairing, was unable to proceed beyond the first stage of meiotic division. Variants of the standard alder pathogen from UK, Germany and the Netherlands exhibited similar irregularities and ranged between

n=13-18, however a variant from Sweden was *n*=11-13 with normal meiosis and fertilisation (Brasier *et al.,* 1999).

Studies of isolates from Hungary found that standard and 'Swedish variant' types of the alder pathogen were both present (Nagy *et al.*, 2003). All isolates from one region (Hévíz) formed oogonia like that of the standard hybrid whereas isolates from another region (Hanság) possessed oogonia typical of the Swedish variants. Furthermore, it was reported that although for the main part oogonia were like one or other of the previously reported alder pathogen types there was further variation beyond that described by Brasier *et al.* (1999) and as such conventional taxonomic methods, as they had suspected, were not sufficient for the classification of *Phytophthora* from alders. Molecular analysis using rRNA ITS RFLP, mtDNA RFLP and RAPDs, including isozyme analysis confirmed that the isolates could be separated into two groups by region that were also akin to standard (Hévíz) isolates and Swedish variant (Hanság) isolates respectively (Nagy *et al.*, 2003).

Hybrid isolates of *P. nicotianae* and *P. cactorum* were also found to be the cause of disease in *Primula* and *Spathiphyllum*. These hybrids were first identified because their cultural characteristics did not fit that of any known *Phytophthora* species. Initially, isozyme analysis the hybrids indicated a three banded pattern with malic enzyme and a three banded pattern with malate dehydrogenase. The fastest band at both enzyme loci co-migrated with the single *P. nicotianae* band, the slowest band co-migrated with the single *P. cactorum* band, and the intermediate was concluded to represent the heterodimeric isozyme. RAPD patterns of the hybrid isolates found that they almost exclusively consisted of bands that were also present in *P. nicotianae* or *P. cactorum*. Southern hybridisation found that bands specific for *P. nicotianae* were present as co-migrating bands in the hybrid isolates. The

same was true for bands of *P. cactorum*. However, mtDNA RFLP patterns only confirmed that *P. nicotianae* was one of the parents (Man in't Veld *et al.*, 1998).

Further molecular characterisation of these natural *P. nicotianae* x *P. cactorum* hybrids found that through PCR analysis using species-specific rRNA ITS primers for *P. nicotianae* and *P. cactorum* that a combination of amplicons typical of each parental species were produced. However, AFLP analysis of hybrid isolates with those of the parental species and two atypical isolates yielded some unexpected results. Consistent differences in AFLP patterns were apparent amongst the hybrid isolates, giving a strong indication that the hybrid isolates may have arisen independently from each other (Bonants *et al.*, 2000).

1.5.2 Basidiomycetes

Hybrid rust fungi were first reported by Spiers and Hopcroft (1994), and were identified by traditional morphological, physiological and ultrastructural features derived from both parental species, *Melampsora larici-populina* and *M. medusae* Thüm., that were found to be present in the hybrid. These species are a serious cause of rust in *Populus* species in Australia and are thought to have entered New Zealand *via* the trans-Tasman wind currents in March 1973. Populations of *M. medusae* were soon restricted to sites where *P. deltoides* cv. Angulata hybrids were growing (Wilkinson and Spiers, 1976). Infection persisted at these locales until 1984 when infection died out. *M. medusae* then remained undetected until infection re-appeared on experimental *P. deltoides* x *P. trichocarpa* hybrid poplars. To overcome severe problems posed by poplar rusts, intensive breeding programmes were started using genetic material imported from around the world to broaden the narrow

genetic base of poplars growing in New Zealand, and wherever possible cultivars resistant to both *Melampsora* species were released. This however was not always possible and soon a 'rust-resistant' poplar cultivar was infected by an apparent hybrid that shared morphological features of *M. larici-populina* and *M. medusae* (Spiers and Hopcroft, 1994). This work is based solely on morphological and cytological data, and it would be useful to know if molecular analysis would reinforce these findings. At present no such work can be found in literature.

Hybridisation has further been described between two species of *Melampsora* (Newcombe *et al.*, 2000; 2001). The natural host of *M. medusae* is *P. deltoides* and of *M. occidentalis* is *P. trichocarpa*. Hybrids of these two poplar species were the mainstay of short-rotation poplar culture in the Pacific North-West USA for decades. One of their benefits was their resistance to leaf rust caused by *M. occidentalis* and *M. medusae*, however, significantly in recent years the hybrid poplar clones have become susceptible to infection. Initially, the cause of infection was found to be *M. medusae* but a new population of leaf rust was found, generated by the hybridisation of *M. medusae* and *M. occidentalis*, species that are believed to have co-evolved with *P. trichocarpa* and *P. deltoides*. As such, their hybrid, *M.* x *columbiana*, combines rust genomes that match those of the dominant hybrid poplar clones.

It was later found that within 14 isolates of *M.* x *columbiana* from the Pacific North-West there were 13 pathotypes when tested on 10 commercial *P. deltoides* x *P. trichocarpa* clones. Conversely isolates of *M. medusae* from the south-east USA could not been differentiated on the same hosts, although they could be differentiated using *P. deltoides* differentials. It was found that pathogenic variation in *M. x columbiana* matched resistance

genes from both *P. trichocarpa* and *P. deltoides* and furthermore revealed the susceptibility of the hybrid poplar species to the hybrid fungus (Newcombe *et al.*, 2001).

The forest pathogen *Heterobasidion annosum* has three intersterility groups that each exhibits a host preference. 'P' is pathogenic to mainly species from *Pinus, Juniperus* and *Calocedrus*; 'S' infects speces from *Picea, Abies, Pseudotsuga, Tsuga* and *Sequoiadendron*; and 'F' has been found only on *Albies alba* in southern Europe (Harrington *et al.*, 1989; Capretti *et al.*, 1990). It was later found that 'S' and 'P' would infect the same host at some sites in California. Through further molecular, isozyme and genetic analysis S x P (SP) hybrids were found, and in one instance in a consortium with S and P on the stump of a ponderosa pine. The authors speculated that the act of felling a tree infected by both S and P intersterility groups provides an effective environment allowing for hybridisation (Garbelotto *et al.*, 1996).

1.5.3 Ascomycetes

The Dutch Elm Disease plagues have involved two predominant species, the first plague by *Ophiostoma ulmi* and the second by *O. novo-ulmi*. These two species differ according to their virulence (the former reportedly less virulent than the latter) and are morphologically and molecularly distinct (Brasier, 1991; Pipe *et al.*, 1995) and in mating tests they are infertile (Brasier *et al.*, 1998). Additionally, *O. novo-ulmi* exists as two distinct races. Firstly, a race thought to have originated in Romania/southern Ukraine and secondly a race from North America (Brasier, 1979; 1990). Both races have spread rapidly but gradually *O. novo-ulmi* has replaced *O. ulmi* populations.

There were probably many opportunities during the first stages of assimilation for interaction as both species occupied the same niche and Brasier *et al.* (1998) found that isolates from two locations (Portugal and Poland) exhibited a combination of mating behaviours and cultural characteristics from *O. ulmi* and *O. novo-ulmi* based on genome-wide molecular approaches (RAPD and RFLP) and on polymorphisms in the gene for the cerato-ulmin toxin. Using RAPD it was found that bands would associate with *O. ulmi* mainly and some with *O. novo-ulmi*, however RFLP of rRNA genes of two isolates showed that they had patterns typical of *O. novo-ulmi* but cerato-ulmin gene sequences typical of *O. ulmi.* The authors concluded that these rare genotypes represent hybrids but that their apparent rarity and low maternal fertility suggest low fitness.

Botrytis aclada is the causal agent of neck rot in onions, a fungal storage disease that can cause severe losses to stored onion crops (Maude and Presley, 1977a, b; Maude, 1988; all as cited by Nielsen and Yohalem, 2001). *B. byssoidea* causes mycelial neck rot in onion, and had been described on the basis of cultural and mycelial characteristics (Walker, 1925; as cited by Nielsen and Yohalem, 2001). Through shared morphological traits, it had been suggested that these two species were conspecific, however, molecular characterisation has distinguished between them. (Nielsen *et al.,* 2001). On the basis of spore size, chromosome numbers and genetic markers *B. aclada* was previously subdivided into two groups AI and AII. Type AI had small spores and 16 chromosomes whereas AII had large spores and 32 chromosomes and it had been suggested that AII was an auto-diploid of the 16 chromosome group (Shirane *et al.*, 1989). Molecular studies using universal-primed PCR (UP-PCR), a method similar to RAPD, distinguished that PCR amplification products of type AII were present in either type AI or *B. byssoidea* but not in both. The authors propose that this result means that AII is a polyploid (amphihaploid in the terminology

used in this thesis) that has arisen through interspecific hybridisation between *B. aclada* (AI) and *B. byssoidea*. It was later proposed that the name *B. allii* is applied to the larger-spored subgroup. Through rRNA gene ITS RFLP and further UP-PCR the authors suggest that the evidence presented shows that the three groups (*B. byssoidea, B. aclada* (AI) and *B. allii* (AII)) are genetically distinct and that isolates of the polyploid species *B. allii* were the results of hybridisation between *B. aclada* and *B. byssoidea* (Yohalem *et al.*, 2003).

1.6 Objectives and proposed studies

1.6.1 Changing of aims

The primary aim of this thesis was to construct artificial amphihaploids via protoplast fusion. It was intended to fuse a *V. dahliae* and a *V. albo-atrum* and analyse the hybrids, and any haploid offspring, both molecularly and pathologically. This aspect, although important in this thesis, was somewhat undermined from the outset by work carried out within the group and reported in Collins *et al.* (2003). In the light of results obtained in these latter studies it was obvious that *Verticillium* crucifer isolates should be considered in a more flexible manner than just that of a basic *V. dahliae* x *V. albo-atrum* hybrid model. The rest of this section shall discuss the implications of the work from Collins *et al.* (2003) and the way that my project was taken forward as a consequence of these results.

1.6.2 Collins *et al.* (2003)

The principal morphologies associated with *Verticillium* crucifer isolates, *V. dahliae* and *V. albo-atrum* divides them into three main types: short-spored/low nuclear DNA and long-spored/high DNA types producing microsclerotia and short-spored/low nuclear DNA producing dark resting mycelium respectively. Within two of these populations both conidial shape and nuclear DNA contents are associated with conidial length. Results from Collins *et al.* (2003) agreed that the majority of crucifer isolates from Europe, Japan, and USA were distinguishable from non-cruciferous isolates by their long spored character. These crucifer isolates produced conidia >7.0 μm in length thus corroborating the data of (Horiuchi *et al.*, 1990; Messner *et al.*, 1996; Karapapa *et al.*, 1997; Zeise and von

Tiedemann, 2001). Five crucifer isolates all from oilseed rape, had their conidial lengths defined as 6.5-6.9 μm and initially were termed as intermediate in her thesis (Collins, 2002) but the definition of long spored was later revised to >6.5 μm to include these isolates (Collins *et al.,* 2003). This revised lower limit for long spored crucifer isolates may be due to chance, reflecting variation within populations or from disparities in measurement.

As mentioned previously, Karapapa *et al.* (1997) reported good correlation for conidial length *versus* nuclear DNA content. It was found from the work of Collins *et al.* (2003) that the long and short-spored isolates formed two discrete populations. Population A, comprised short-spored low DNA *V. dahliae* isolates, *V. albo-atrum* and two *Verticillium* crucifer isolates. Only *Verticillium* crucifer isolates were found in population B formed of isolates with the long-spored high DNA characteristics. However, it cannot be over emphasised that two discrete populations are formed with no isolates having intermediate properties.

AFLP was used to define the relationships at a whole genome level between *Verticillium* crucifer isolates and *V. dahliae*, *V. albo-atrum* (L) and *V. albo-atrum* (NL). For each group of isolates studied, AFLPs clearly differentiated between them. Within *V. dahliae* a significant level of variation was observed but this was not apparent within either pathotypes of *V. albo-atrum*. This result agreed with earlier results of RFLP (Carder and Barbara, 1991). AFLP analysis produced two distinct clades within the long-spored *Verticillium* crucifer isolates, α and β respectively, with a restricted amount of variation observed within these two sets. Group β is further subdivided on the basis of rRNA gene type, demonstrated by having either *V. dahliae*-like or *V. albo-atrum*-like ITS sequences.

Further subdivision is allowed for through identification of ITS sequence variants and the retention of a minor type rRNA gene sequence. This AFLP group may also be divided on the basis of mtDNA type. However, few isolates are found to comprise this group but this may be due to bias introduced through sampling, and it requires more isolates to define the variability seen within it. AFLP group α is seemingly more homogeneous. Collins *et al.* (2003) proposes that the critical interpretation of this result is that long spored crucifer pathogenic interspecific hybrids have arisen on at least two separate occasions, represented by the two AFLP groups. α comprises the crucifer isolates from Europe, Japan, USA and Russia. AFLP and other molecular data (ITS, IGS, mtDNA RFLP) implies that these are of clonal origin and the occurrence of this group on different continents suggests that it has been spread on imported propagation material or infected plants.

Group β represents the second hybridisation event and comprises horseradish isolates from Europe (*V. dahliae* var. *longisporum*) and USA and one European oilseed rape isolate. This hybridisation event is not as prominent within Europe as group α but is the cause of substantial losses in horseradish in USA. The subdivisions observed within the β group may be explained through the loss/selection of highly duplicated genes and mitochondria. The crucifer isolates within groups α and β were more similar to *V. dahliae* than either *V. albo-atrum* pathotypes, thus corroborating the RAPD results of Karapapa *et al.* (1997). The latter authors suggested that European oilseed rape isolates and some from crucifers isolates from Japan had arisen from a hybridisation event with a *V. albo-atrum* (L) isolate. This conclusion was attributed to a number of the RAPD bands found were present solely in isolates of *V. albo-atrum* (L). Results from Collins *et al.* (2003) showed that the *V. albo-atrum* Lucerne and Non-Lucerne specific AFLP bands were not present in the same proportion as the *V. dahliae* bands in groups α and β. This implies that neither *V. albo-*

atrum pathotypes was likely to be the parental isolates involved in these hybridisation events.

As previously discussed two short-spored *Verticillium* cruciferous isolates fell into the short-spored low DNA content category. Through AFLP analysis it is apparent that they are distinct both from *V. dahliae* and *V. albo-atrum,* and from each other. It was suggested that these are derived from interspecific hybrid isolates and would be considered as 'secondary haploid'. They are, in comparison to amphihaploids, closely allied with *V. dahliae* isolates thus implying that a major part of the parental *V. dahliae* genome is retained through rehaploidisation but their discrete grouping also suggests that some smaller part of the other parental genome has been retained.

1.6.3 New aims

This project aimed to construct viable interspecific hybrids of *V. dahliae* and *V. albo-atrum* through protoplast fusion without utilising mutagens to induce auxotrophic mutants in the parents. The origins and relationships of amphihaploids and secondary haploids were to be investigated through molecular, morphological and pathological analyses of artificial amphihaploids and secondary haploids derived from them. As discussed previously, extensive analyses of a wide range of cruciferous isolates alongside *V. dahliae* and *V. albo-atrum* by a range of molecular methods demonstrates that we have not seen at least one of the parents in both supposed hybridisation events. It may simply be that the isolates involved in the hybridisation events may be of ancient origin and they may no longer be in existence, but it was clear that for the immediate remit of this project the construction of artificial amphihaploids had little to gain as they may not reflect the relevant event.

However, continuing analysis of *V. dahliae* and *V. albo-atrum* isolates by methods already utilised and potential new markers may add some light into the matter.

As mentioned previously, it was necessary to consider *Verticillium* crucifer isolates in a broader manner than that of *V. dahliae* x *V. albo-atrum* hybrids. It has always been assumed through some circumstantial and limited evidence by many authors that the *Verticillium* crucifer isolates are *V. dahliae* x *V. albo-atrum* hybrids. By returning to the ITS it can be seen that at least one related weak pathogen, an isolate of *V. nubilum*, is as close to the majority of crucifer isolates as *V. dahliae* (Zare *et al.*, 2000). Extending the range of isolates studied to related species such as *V. nubilum*, *V. theobromae* and *V. nigrescens* might provide answers to some of the questions posed by results from AFLP, ITS and IGS studied that are not explained by the *V. dahliae* x *V. albo-atrum* hypothesis. Once a possible parent or the nearest isolate to a possible parent is found then it may prove beneficial to return to the hybridisation studies that were laid out in the initial aims.

Furthermore it is deemed necessary to carry out pathogenicity tests on cruciferous crops of a range of *Verticillium* crucifer isolates, *V. dahliae*, and *V. albo-atrum* plus some related species to determine whether the long-held assumption that isolates of *Verticillium* from crucifers are more virulent to cruciferous crops than *V. dahliae* and *V. albo-atrum*, or even host-specific, is true.

Finally, the implications of these crucifer isolates will be discussed as to their implications on their taxonomy and that of other plant pathogens, including the effect on the practice of plant pathology.

2 GENERAL MATERIALS AND METHODS

2.1 Media

The pH of all media was adjusted to 5.8 with 1M NaOH and sterilised at 121°C (15 psi for 15 minutes. All amounts are per litre of solution unless otherwise stated.

2.1.1 Basal Medium (BM) (Correll *et al.*, 1987)

$FeSO_4.7H_2O$	10 mg
$MgSO_4.7H_2O$	500 mg
KCl	500 mg
KH_2PO_4	1 g
Sucrose	30 g

modified with 200 μl trace element solution consisting of the following substances:

Trace Element Solution for Basal Medium (Correll *et al.*, 1987)

(per 95ml of distilled H_2O)

Citric acid	5 g
$ZnSO_4.7H_2O$	5 g
$Fe(NH_4)_2(SO_4)_2.6H2O$	1 g
$CuSO_4.5H_2O$	250 mg
$MnSO_4.H_2O$	50 mg
H_3BO_4	50 mg
$NaMoO_4.2H_2O$	50 mg

Minimal medium (MM) was made by adding 2 g of $NaNO_3$ to 1 litre of basal medium

BM with hypoxanthine was made by adding 0.5 g of hypoxanthine to BM.

For BM agar, MM agar or BM with hypoxanthine agar add 10 g Oxoid agar technical (Agar No. 3) per litre of medium

2.1.2 Prune Lactose Yeast-extract medium (PLY) and Prune Lactose Yeast-extract Agar (PLYA) (Talboys, 1960)

Sunsweet prune juice	10 ml
Lactose	5 g
yeast extract	1 g

For PLYA, add 10 g Oxoid agar technical (Agar No. 3) per litre

2.2 Fungal cultures and maintenance

Plant pathogenic *Verticillium* isolates were imported and maintained under the conditions of UK DEFRA licence PHL 166D/4968 and renewals. All isolates received during the course of this PhD were initially maintained on PLYA in the dark at 20-25°C. For long-term storage conidial suspensions were prepared by washing the surface of a plate with 1 ml sterile distilled water. Cryovials containing *ca.* 15 beads, of diameter 3mm (Creative Beadcraft Ltd, Amersham, UK) were filled with 75 μl of conidial suspension and 75 μl of 20% glycerol in aqueous solution and stored in a -80°C freezer.

When required, mono-conidial isolates were prepared. Spore suspensions were prepared as above, the concentration determined using a haemocytometer and diluted to give a concentration of 4×10^4 per ml. Suspensions were further diluted to give 200 spores per

ml, and from this 200 μl was spread over the surface of a PLYA plate. After 24 hours of incubation in the dark at 20-25°C, conidia could be seen to have germinated using a binocular microscope and individual colonies were picked off aseptically and grown individually on PLYA.

2.3 DNA extraction methods

2.3.1 Squash Blot

Chromatography paper (3MM; Whatman International Ltd, Maidstone, UK) was laid onto a glass plate, soaked with 1M NaOH and small squares of NytranN (Schleicher&Schuell Biosciences GmbH, Germany) placed onto the chromatography paper. Using aseptic technique, mycelium was removed and crushed against the membrane using a disposable stirring rod (Sarstedt, Leicester, UK). The membranes were then transferred individually to a small weighing boats, rinsed with 1.5M NaCl, 0.5M Tris, 1mM EDTA, pH 7.0, three times with 1 x TE and finally with RO water. To elute the DNA, the membrane segments were pushed into 0.5 ml microfuge tubes, 60 μl of RO water added and the tubes heated at 94°C for 5 minutes. The DNA was stored at -20°C until required.

2.3.2 DNeasy Plant Mini Kit and DNeasy Plant Maxi Kit

Conidia were washed from the surface of PLYA plates with 1 ml sterilised RO water and diluted to give 1 x 10^6 spores per ml. 1 ml of each conidial suspension was added aseptically to 125 ml of sterile PLY medium in 250 ml conidial flask. The flasks were incubated in an orbital shaker maintained at 23-25°C and 100 rpm for 7 days. Mycelium was harvested by filtering the culture through two layers of muslin, rinsing in RO water

and blotting dry. The wet weight of each isolate was recorded and mycelia frozen overnight at -20°C before lyophilisation over 2-4 days. For the DNeasy plant mini and maxi kits, 30 mg and 200 mg respectively of lyophilised mycelium was ground with sterilised sand, and processed in accordance with manufacturers' instructions. Genomic DNA was stored at -20°C until required.

2.4 PCR amplification

DNA amplifications were performed in a total volume of 25 μl consisting of either 5 μl squash blot DNA or 10 ng of genomic DNA, 10 pmol of each primer (Invitrogen) (Table 2.1) 0.2mM of each dNTP (Amersham Pharmacia Biotech), 1.5mM $MgCl_2$, 1 x PCR buffer (50mM KCl, 20mM Tris-HCl, pH 8.4) and 0.65 U *Taq* polymerase (Invitrogen). All amplifications were performed in a FlexiGene thermal cycler (Techne (Cambridge) Ltd, UK). After an initial denaturation (93°C/2min) and annealing step (the appropriate annealing temperature for the primer pair/15sec) reactions were given 40 cycles (72°C/30sec, 93°C/15sec, the appropriate annealing temperature for the primer pair /15sec) with a final 72°C/10min.

Gene or region	Primer number	Primer name	Sequence (5'-3')
ITS	219	ITS4[a]	TCC TCC GCT TAT TGA TAT GC
	220	ITS 5[a]	GGA AGT AAA AGT CGT AAC AAG G
	361[b]		CCG GTA CAT CAG TCT CTT TA
	363[b]		CCG GTC CAT CAG TCT CTC TG
IGS	723[c]		CAC GGA ACC AAC TCT GCC
	727[c]		TCC TAC CAT CTA TGG AGG
5*S* rRNA IGR	846[d]		AGT AGT YGG GTG GGT GAC CA
	847[d]		GGG AGA GCG GAC GGG ATC
Non-defoliating and defoliating pathotype	858	INTD2f[e]	ACT GGG TAT GGA TGG CTT TCA GGA CT
	859	INTD2r[e]	TCT CGA CTA TTG GAA AAT CCA GCG AC
	860	INTND2f[e]	CTC TTC GTA CAT GGC CAT AGA TGT GC
	861	INTND2r[e]	CAA TGA CAA TGT CCT GGG TGT GCC A
β-tubulin gene	943	btubT1[f]	AAC ATG CGT GAG ATT GTA AGT
	944	btubT22[f]	TCT GGA TGT TGT TGG GAA TCC
	945	btubT11[f]	AAT TGG TGC TGC TTT CTG GCA
	946	btubT2[f]	TAG TGA CCC TTG GCC CAG TTG
Mitochondrial cytochrome B gene	955[d]		TTC RTW TGA GGD GGT TTY WSH G
	957[d]		GCR TAG AAW GGT AAT AAR TAT
5*S* rRNA gene	958[d]		TCA TAC AAC ACC AGG GAT TCG
	959[d]		TCC ACA TAC GAC CAT ACC TAT
AFLP	651	*Eco*RI-A[g]	GAC TGC GTA CCA ATT CA
	652	*Eco*RI-C[g]	GAC TGC GTA CCA ATT CC
	653	*Eco*RI-G[g]	GAC TGC GTA CCA ATT CG
	654	*Eco*RI-T[g]	GAC TGC GTA CCA ATT CT
	655	*Mse*I-CA[g]	GAT GAG TCC TGA GTA ACA
	656	*Mse*I-CT[g]	GAT GAG TCC TGA GTA ACT
	657	*Mse*I-CC[g]	GAT GAG TCC TGA GTA ACC
	658	*Mse*I-CG[g]	GAT GAG TCC TGA GTA ACG

Table 2.1. Primers used in this thesis. Source of primers: [a] White *et al.* (1990), [b] Nazar *et al.* (1991), [c] Collins *et al.* (2003), [d] designed during this study, [e] Mercado-Blanco *et al.* (2001, 2002), [f] O'Donnell and Cigelnik (1997), [g] Invitrogen. 'Primer number' is the reference to the catalogue of primers in Dr Dez Barbara's lab at Warwick HRI.

3 HYBRIDISATION STUDIES: *V. DAHLIAE* X *V. ALBO-ATRUM*

3.1 Introduction

Plant pathogenic *Verticillium* are classified as Fungi Imperfecti with no recorded teleomorph (or sexual stage) found. The origin of *V. dahliae*, was however placed firmly within the Ascomycetes by complete sequencing of the 18*S* rRNA gene complex and comparative phylogenetic analysis (Messner *et al.*, 1996). If it is assumed that a sexual cycle is not operative, it must be concluded that the sources of genetic diversity within the plant pathogenic *Verticillium* species are gene mutation, possibly internal recombination, or parasexual recombination. The normal parasexual cycle consists of the formation of diploid nuclei by nuclear fusions in heterokaryons followed by the occurrence of mitotic crossing-over and haploidisation of the diploid nuclear lineage.

The underlying principles of parasexual genetics were uncovered by the work of Alec Hastie in the 1960s-1970s. This work had important repercussions for not only *Verticillium*, but for recombination in all asexual fungi. In *Verticillium*, parasexual recombination was thought to give only haploid isolates and the concept of isolates of *Verticillium* from crucifers as hybrids is only recent as is our understanding of their distinct genetics.

3.1.1 The parasexual cycle and *Verticillium*

Hyphal anastomosis and the formation of heterokaryons is necessary for parasexual processes; the fusion of haploid homokaryotic hyphae coupled with restricted nuclear

migrations results in the formation of haploid heterokaryotic mycelium. Isolated somatic nuclear fusion may occur between haploid heterokaryons to form diploid nuclei, which may be heterozygous at the complementary gene loci of the original homokaryon. If they have a selective advantage, the heterozygous diploids will multiply but genetic recombination may occur and non-disjunction result in unstable novel diploid segregants. The progressive loss of chromosomes results in mycelium containing novel recombinant haploids, the parental homokaryon types, and heterozygous aneuploid and diploid nuclei (Pegg and Brady, 2002).

In *Verticillium*, parasexual events were first reported in isolates of *V. albo-atrum* from hops with genetic recombination demonstrated between the nutritional mutants. It was also noted that 'the most striking feature of the genetic recombination is the relative frequency at which it occurs' (Hastie, 1962).

In a later study by the same author (Hastie, 1964) it was found that 'diploid' isolates were unstable, yielding haploid isolates readily, usually within three weeks. Mitotic crossing-over was also an important feature of these crosses which occurred frequently, producing homozygous and heterozygous segregants for the markers used. Crossing-over was also detected by changes in linkages of markers during vegetative growth. Some heterozygous segregants were either aneuploids, or were formed by double mitotic crossovers. Haploid segregants, derived from nuclei that had previously undergone mitotic-crossing over were often recovered and this link of mitotic crossing-over and haploidisation in one nuclear lineage, along with the possible occurrence of double mitotic crossovers meant that mitotic analysis was less clear cut than in *Aspergillus nidulans* an organism that was also being studied extensively at the time (Hastie, 1964).

Later work included *V. dahliae* as well as *V. albo-atrum*. Auxotrophic mutants of *V. albo-atrum* Lucerne isolates were found to produce heterokaryons when complementary pairings were brought together, however this was not the case for pairings of isolates from tomato and Lucerne *V. albo-atrum* or for *V. dahliae* x *V. albo-atrum* Lucerne isolates. It was clearly found that cytoplasmic inheritance was possibly linked to morphogenic factors such as the production of a dark pigment *i.e.* melanin. Furthermore, nuclear migration was found to occur through the anastomosing germ tubes of uninucleate conidia. Heterokaryotic mycelium is a mosaic, the margins of which are unstable with imbalanced nuclear ratios (Heale, 1966; 1988).

It is thought that only isolates belonging to the same vegetative compatibility group (VCG) are able to undergo hyphal anastomosis (Figure 3.1) to produce viable heterokaryons, the first step in the parasexual cycle. Vegetative compatibility is a useful marker for determining the genetic structure in fungi such as *Verticillium*, as isolates belonging to the same VCG are generally more genetically similar to each other than they are to isolates in other VCGs.

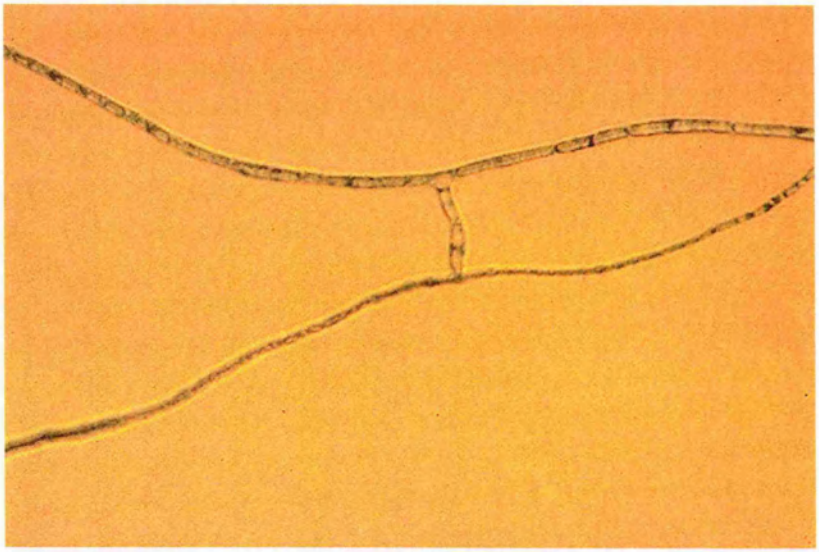

Figure 3.1. Two fungal hyphae of *Verticillium* with an anastomosis bridge between them. These two hyphae belong to the same vegetative compatibility group. Courtesy R. Rowe http://www.scisoc.org/offline/lessonimagereview/verticillium/imagereview.htm

3.1.2 Vegetative compatibility groups in plant pathogenic *Verticillium*

In *V. dahliae*, six VCG have been identified from a range of geographical locations and hosts (Joaquim and Rowe, 1990, 1991; Strausbaugh *et al.*, 1992; Chen, 1994; Bhat and Subbarao, 1999; Elena and Paplomatas, 1998; Elena 1999a, b; Korolev *et al.*, 2000; Douhan and Johnson, 2001; Goud and Termorshuizen, 2002; Cherrab *et al.*, 2002; Bhat *et al.*, 2003; Tsror and Levin, 2003; Chandelier *et al.,* 2003). Further sub-division of groups has been described in VCGs 2 and 4 based on differential interactions among tester strains within each group (Joaquim and Rowe, 1991; Strausbaugh, 1993). For the main part, VCG does not correlate with host pathogenicity but there does seem to be some correlation with virulence on certain hosts. VCG 4 is predominant in potato populations of *V. dahliae* (Joaquim and Rowe, 1991; Strausbaugh *et al.*, 1992) but isolates belonging to VCG 4A are more virulent than VCG 4B (Joaquim and Rowe, 1991; Strausbaugh, 1993; Douhan and

Johnson, 2001). Disease caused by isolates from VCG 4A is exacerbated further by a synergistic interaction with root lesion nematode *Pratylenchus penetrans* (Botseas and Rowe, 1994.

Molecular analysis of VCG 4A and 4B has revealed the existence of two genetically distinct types of VCG 4A in North America (Dobinson *et al.*, 2000). Potato isolates from Israel were placed into VCG 4B and were found to be more aggressive than isolates from either VCG 2A and 2B (Tsror *et al.*, 2001). Isolates from the defoliating pathotype of cotton in the US and Spain have been found to be in VCG 1 whereas isolates of the non-defoliating pathotype fell into VCG 2 and VCG 4 (Joaquim and Rowe, 1990; Daayf *et al.*, 1995; Korolev *et al.*, 2001). Cotton isolates from Israel that cause severe wilting and partial defoliation belong to VCG 2B whereas non-defoliating isolates fell into VCG 2A and 4B (Korolev *et al.*, 2001).

Two VCGs have been identified in *V. albo-atrum* that corresponds to the Lucerne and Non-Lucerne pathotypes. All isolates from Lucerne fall into VCG 01 whereas Non-Lucerne infecting isolates fall into VCG 02 (Correll *et al.*, 1988; Furgal-Wegrezyca, 1997). These studies along with molecular characterisation confirm that *V. albo-atrum* isolates from Lucerne consist of a genetically homogenous, probably clonal population, distributed worldwide.

3.1.3 Interspecific complementation

Questions concerning genetic recombination and *V. dahliae* x *V. albo-atrum* hybrids were posed before the nature of *V. dahliae* var. *longisporum* as a natural interspecific hybrid was

considered. Hastie (1973), using auxotrophic mutants of *V. dahliae* and *V. albo-atrum*, induced the formation of interspecific hybrids. That author reported that only limited recombination occurred when interspecific hybrids gave rise to rare spontaneous haploid isolates thus indicating non-homology between the parental genomes. The author comments that the justification for defining *V. dahliae* and *V. albo-atrum* as separate species may seem 'illogical' with regard to the apparent ease that hybrids were formed under selective conditions. Later studies (Typas, 1983) somewhat contradicted Hastie (1973) and suggested that the genetic segregation among haploid isolates derived from interspecific hybrids grown in the presence of haploid-inducing agents show that the two species are closely allied and that substantial chromosome homology may occur between them. In these studies, the authors produced *V. dahliae* x *V. albo-atrum* (in addition to intraspecific hybrids) via protoplast fusion and microinjection, thus circumventing the effects of heterokaryon incompatibility that may be mediated at the cell wall.

Removing the barriers of heterokaryon incompatibility, via microinjection or protoplast fusion allows for extensive interaction between any two isolates (almost certainly with more ease than with which it occurs in nature) and also permits a reliable artificial system for comprehensive genetic and molecular analysis.

3.1.4 Aims and Objectives

- Pair complementary *nit* mutants of *V. dahliae* and *V. albo-atrum.*
- Analyse progeny derived from mycelial interface for *nit* phenotype, rRNA ITS and by AFLP analysis.
- Develop effective method of purification of protoplasts suitable for both PEG-mediated fusion and electrofusion protocols
- Create artificial hybrids using protoplast fusion
- Analyse artificial hybrids for phenotype and use molecular markers rRNA ITS and AFLP to assess extent of genetic recombination.

3.2 Methods

3.2.1 Interspecific *nit* mutant pairings

3.2.1.1 Complementation of *nit* mutants of *V. dahliae* and *V. albo-atrum*

Microsclerotia were obtained from the interface of pairings between *V. albo-atrum* (KRS1) *nit1* x *V. dahliae* (115) *nitM* and *V. albo-atrum* (STR3) *nit1* x *V. dahliae* (115) *nitM* on MM. Irregular (*i.e.* subjectively selected as being irregularly shaped) microsclerotia were removed and subcultured on to MM or PLYA (see 2.1) (Cultures 1-27). Cultures 1-15 were grown on PLYA and the functional rRNA gene repeat ITS sequence determined by PCR amplification using primer combinations ITS4/361 and ITS4/363 with the identity of the amplicon verified by *Hae*II restriction digest analysis (Collins, 2002). Monoconidial cultures were prepared from cultures 11 and 12. Twenty single spores were taken from each culture. The complementation of interspecific *nit* mutants on MM, the removal of irregular microsclerotia and verification of minor type functional rRNA gene ITS of cultures 1-15 and *Hae*II restriction digest were all undertaken by Alexandra Collins, Horticulture Research International, Wellesbourne, UK.

3.2.1.2 Monoconidial cultures derived from cultures 11 and 12

From each of the twenty monoconidial cultures obtained from cultures 11 and 12, DNA was extracted using the squash blot method as previously described (see 2.3). The functional rRNA gene ITS sequence was determined by PCR amplification using primers ITS4/361 and ITS4/363.

3.2.1.3 *nit* phenotype of twenty monoconidial isolates derived from cultures 11 and 12

The *nit* phenotype of the twenty monoconidial isolates derived from each of cultures 11 and 12 was determined by subculturing each isolate onto MM and BM supplemented with hypoxanthine.

3.2.1.4 AFLP analysis of twenty monoconidial isolates from each of cultures 11 and 12

AFLP analysis was carried out on 250 ng of DNA using the AFLP Analysis System II kit (Invitrogen). RE digestion, ligation of adaptors and pre-amplification were carried out in accordance with the manufacturer's instructions. Selective amplification was carried out using *Mse*I primers containing two selective nucleotides and *Eco*RI primers containing one selective nucleotide (Table 2.1). PCR conditions were as follows: 1 x PCR buffer (50 mM KCl, 20 mM Tris-HCl, pH 8.4), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 30 ng of each primer, 0.5 U of *Taq* polymerase and 5 μl of diluted (1:50) pre-amplification product in a final volume of 20 μl. The PCR cycling parameters was 30s at 94°C, 30s at 65°C and 60s at 72°C for 13 cycles with the annealing temperature lowered by 0.7°C each cycle) followed by 23 cycles of 30s at 94°C, 30s at 56°C and 60s at 72°C. Three μl of the selective amplification product was loaded onto a Spreadex 800 gel (Elchrom Scientific) and separated by electrophoresis at 50-55°C for 2h at 120V. Spreadex gels were stained with SYBR Gold diluted 1:10000 in 0.33 x TAE for 45 mins and destained in RO water for 2 hours. Bands were visualised and recorded using the Imago image analysis system. The DNA profiles generated from different primer combinations were scored manually as follows: 1 if a specific molecular weight band was present and 0 if it was absent. The data

was analysed using Genstat (Version 6, Lawes Agricultural Trust, Rothamsted, UK) using an UPGMA approach and Jaccard's coefficient. Results from cluster analysis are presented as a dendrogram to display genetic distance between isolates.

3.2.2 Protoplast generation and purification

Protoplasts were prepared from 5 different isolates, three of *V. dahliae* (1877, 12078, 12080) and two of *V. albo-atrum*, one of each pathotype (Lucerne-STR3, Non-Lucerne-1844). Three methods of purification were studied.

3.2.2.1 Protoplast generation

Protoplasts were generated by a method adapted from Okoli (1992): 125ml of PLY medium (see 2.1) was autoclaved for 15 minutes as 121°C in 250ml conical flasks. Spore suspension, prepared by washing the surface of a culture on PLYA (see 2.1), was inoculated at a final concentration of 1 x 10^4 spores per ml into the autoclaved PLY medium. Cultures were incubated in an orbital shaker maintained at 20-25°C, 100rpm for 36-40 hours. Mycelia from each isolate was harvested by centrifugation at 5343 x *g* for 10 minutes, and washed by resuspending in 0.6M $MgSO_4$. For methods utilising Percoll® (Amersham Pharmacia Biotech UK Limited, Little Chalfont, Buckinghamshire, UK) mycelium was washed using 0.6M sorbitol. The supernatant was discarded and washing repeated as above to remove mucilaginous material.

Harvested and washed mycelium from each isolate was re-suspended in 10 ml of an enzyme mixture containing 5mg/ml Lysing Enzyme from *Trichoderma* sp. and 5mg/ml Driselase (Sigma-Aldrich Company Ltd., Poole, Dorset, UK) (Personal communication Dr.

Adriana Soares, Warwick HRI) prepared in the required osmotic buffer (see results) that had been filter sterilised (Millipore, 0.22 μm). The mycelium with the enzyme mixture were incubated in an orbital shaker at 30°C, 80 rpm for 150-180 minutes.

3.2.2.2 Purification of protoplast suspension utilising an interface between 1.2M $MgS0_4$ and 600mM sorbitol, 100mM tris.HCl pH 7.0

The same enzyme mixture as above was prepared with 1.2M $MgSO_4$. Post-incubation the enzyme digest/mycelium mixture was passed through two layers of sterile muslin. The resulting liquor was overlaid with 600mM sorbitol, 100mM tris.HCl, pH 7.0 and centrifuged and 5343 x *g* for 30 minutes in a swing-out rotor. The best rate of fixed linear acceleration curve of the centrifuge, and the slope of the linear de-acceleration curve, was briefly studied to determine the most effective rate of increase/decrease in speed to maintain the interface of the two liquids. The rate of acceleration/de-acceleration was set at 180 seconds per 1000rpm.

Protoplasts gathered at the interface between the two liquids whilst mycelial debris and conidia pelleted. Protoplasts were then transferred to a fresh sterile tube, dispersed in an equal volume of 600mM sorbitol, 10mM tris.HCl, pH 7.5 and pelleted by centrifugation at 3577 x *g* for 15 minutes. Protoplasts were washed twice more in the same solution and finally re-suspended in 1 ml of 600mM sorbitol, 100mM EDTA, pH 8.0 solution (Okoli, 1992).

3.2.2.3 Purification of protoplast suspension using 50% Percoll

The enzymes were prepared in 800mM sorbitol, 10mM tris.HCl pH 7.0 and after incubation the enzyme-digest mix/mycelium were passed through two layers of sterile muslin.

The removal from the protoplast suspension of undigested mycelium and spores was tested by the following procedure; 1. adding Percoll prepared with 800mM sorbitol, 10mM tris.HCl to a final concentration of 50% so that the mycelia was digested in the presence of Percoll, 2. adding Percoll to a final concentration of 50% after the enzyme digestion and 3. adding Percoll prepared with 800mM sorbitol to a final concentration of 50%, and was added in equal volume after the mycelial digest to a final concentration of 25% Percoll.

The enzyme digest mixture was passed through two layers of sterile muslin and the resultant liquor was overlaid carefully with an equal volume of 600mM sorbitol, 100mM tris.HCl pH 7.0 and centrifuged at 5343 x *g* for 30 minutes. The rate of acceleration/de-acceleration was set at 180 seconds per 1000 rpm. Protoplasts gathered at the interface between the two solutions whilst the mycelial debris and conidia pelleted. The protoplasts were transferred to a fresh sterile tube and re-suspended in an equal volume of 600mM sorbitol, 10mM tris.HCl, pH 7.5 and pelleted by centrifugation at 3577 x *g* for 15 minutes. Protoplasts were washed twice further and re-suspended in 1 ml of 0.6M sorbitol.

3.2.2.4 Purification of protoplasts using 15% and 25% Percoll

For separation of protoplast suspension from undigested mycelia and conidia, the protocol that follows was modified from a method used for purification of protoplast of tomato (*Lycopersicon esculentum*) (Fieuw and Willenbrink, 1991).

The enzyme-digest/mycelium mixture was passed through two layers of sterile muslin. The resultant liquor was centrifuged at 2780 rpm for 15 minutes. The pellet was re-suspended either in 15% Percoll, 0.6M sorbitol solution or 25% Percoll, 0.6M sorbitol solution and centrifuged in a swing-out rotor at 5343 x *g* for 20 minutes with the rate of acceleration/de-acceleration set at 180 seconds per 1000 rpm. Protoplasts that collected at the meniscus layer were transferred to a sterile tube and dispersed in the required Percoll, 0.6M sorbitol solution. An equal volume of 0.6M sorbitol was carefully overlaid onto the protoplast suspension and centrifuged at 3080 x *g* with the rate of acceleration/de-acceleration at 180 seconds per 1000 rpm. Protoplasts found at the interface between the two liquids were removed and dispersed in 0.6M sorbitol and centrifuged at 4260 x *g* for 15 minutes. The pellet was re-suspended in 1 ml of 0.6M sorbitol.

3.2.2.5 Single carbon substrate utilisation profiles of three *Verticillium* isolates

Spore suspensions were prepared from three isolates of *Verticillium* (12078, STR3, VdII) by washing the surface of a monoconidial culture on PLYA. The API 50 CH strip (bioMérieux (UK) Ltd., Basingstoke, UK) was prepared as described in the manufacturer's instructions except spores were inoculated in the microtubules at 1 x 10^4 per ml. The strip was incubated at 20-25°C in the dark until growth was visible in some microtubes. After incubation each microtube was examined under a binocular microscope and scored on an

arbitrary scale –2 to +3 relative to observed growth in the control tube which was scored as 0 (and which had no added carbohydrate substrate).

3.3 Results

3.3.1 Interspecific *nit* mutant complementation

3.3.1.1 Mono-conidial cultures derived from cultures 11 and 12

From cultures 11 and 12, each which have been derived from a single microsclerotium, 20 mono-conidial isolates were prepared from each and the functional rRNA gene ITS type determined via PCR amplification using primers ITS4/361 for *V. albo-atrum*-like ITS type and ITS4/363 for *V. dahliae*-like ITS type.

From culture 11, PCR amplification showed that nine mono-conidial isolates had *V. dahliae*-like ITS and eight mono-conidial isolates had *V. albo-atrum*-like ITS. Three mono-conidial isolates (11c, 11r, and 11s) gave both *V. dahliae* and *V. albo-atrum* ITS types in the first PCR amplification. The amplification was repeated using fresh DNA prepared using the squash blot method and this showed that 11c carried the *V. albo-atrum* ITS type and 11r and 11s of *V. dahliae* ITS-type.

PCR amplification of the functional rRNA gene ITS regions of squash blot DNA from 20 mono-conidial isolates prepared from culture 12 showed that five had *V. dahliae*-like ITS, fifteen had *V. albo-atrum*-like ITS.

3.3.1.2 *nit* phenotype of twenty mono-conidial isolates derived from cultures 11 and 12

Each of the twenty mono-conidial isolates derived from each of the cultures 11 and 12 were subcultured onto MM and BM supplemented with hypoxanthine to determine the *nit* phenotype of each mono-conidial isolate.

All mono-conidial isolates derived from culture 11 were shown to have the *nit* phenotype which corresponded to that of the ITS type in the 'parental' isolates. From the mono-conidial isolates prepared from culture 12, nineteen isolates had corresponding *nit* phenotype to ITS type. Isolate 12.20 was shown to have a novel combination of phenotypes, *V. albo-atrum* STR3 derived *nit*1 with *V. dahliae* 115 derived ITS.

3.3.1.3 AFLP analysis of twenty mono-conidial isolates from each culture

DNA from 40 mono-conidial cultures derived from each of the two cultures each produced from a single microsclerotium excised from the mycelial interface of an interspecific *nit* mutant pairing was subjected to AFLP analysis. DNA fragments ranging from 100 bp to 500 bp were resolved using Spreadex 800 gels followed by SYBR gold staining (Figure 3.2). Of 16 primer combinations used, 14 produced informative AFLP profiles for statistical analysis. Fingerprints from 14 primer combinations were used to generate a dendrogram (Figure 3.3a) to examine the relationships of the mono-conidial isolates to the 'parental' isolates. AFLP produced 220 scorable bands, 180 of which discriminated between the parental *V. dahliae* (103 bands) and *V. albo-atrum* species (77 bands). There

were 28 bands common to both parental species, and 12 that were only found in the progeny isolates.

Two main clades were formed, with the 'parental' *V. dahliae nit* mutant isolate (115) in a separate clade from that containing the *V. albo-atrum nit* mutant isolate (STR1) separated in each clade. For the main part, isolates that clustered with the *V. dahliae* or *V. albo-atrum* 'parental' isolates corroborated results from their assessment of *nit* phenotype and rRNA ITS type. Isolate 12.20, that showed novel combination of rRNA ITS and *nit* phenotype clustered with isolates like that of the *V. albo-atrum* parent.

In order to increase the apparent discrimination the analysis was repeated using AFLP profiles that generate a score of 0 or 1 respectively in either parent, but not when a score of 0,0 or 1,1 was observed (Figure 3.3b). As expected while different in detail the results revealed a very similar picture as in shown in Figure 3.3a.

According to the AFLP analysis, none of the 'progeny' isolates were identical to either parent, with isolate 11m as divergent from the other *V. dahliae* isolates as STR3 is from the *V. albo-atrum* isolates (Figure 3.3a,b). Limited recombination between the parental genomes was apparent at some loci (Table 3.1). Within the *V. albo-atrum*-like AFLP clade it was shown that three primer combinations (651/655, 651/658, 652/658) had the *V. dahliae* derived polymorphisms consistently in 16 isolates out of 25. Within the *V. dahliae*-like AFLP clade two primer combinations (651/655, 652/658) produced a consistent amplification of a *V. dahliae* polymorphism in 8 isolates of a total 14 isolates, and another polymorphism was found using two primer combinations (652/658, 653/655) in an

additional four isolates. The combinations of rRNA gene ITS type, *nit* phenotype and AFLP group are summarised in Table 3.2.

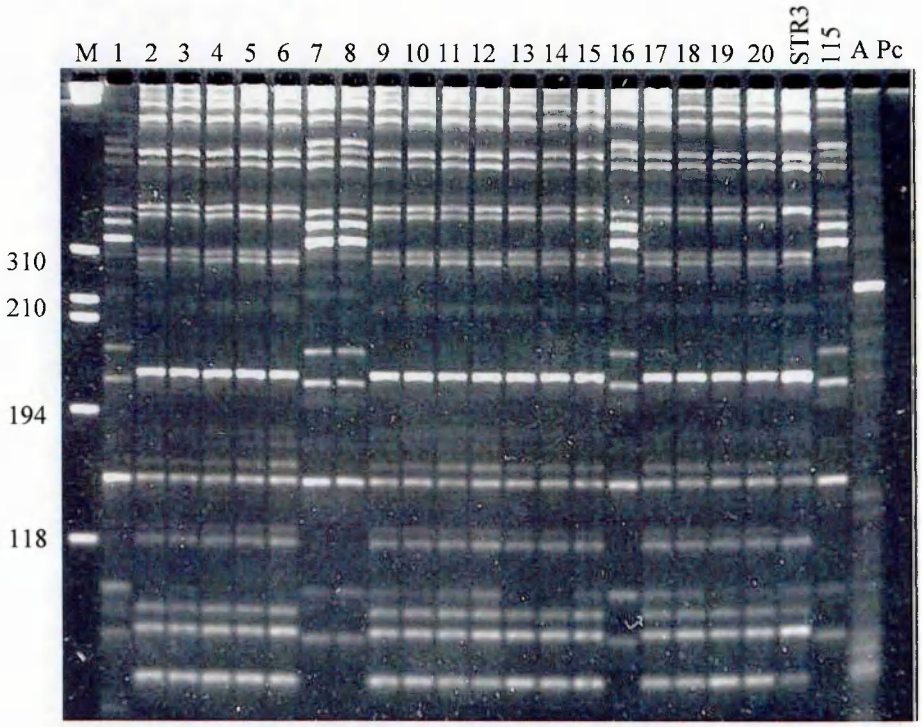

Figure 3.2. Separation and visualisation of AFLP bands on a Spreadex 800 gel. Monoconidial isolates from culture 12, 1-20. M: Molecular marker; A: Arabidopsis control; Pc: Primer control

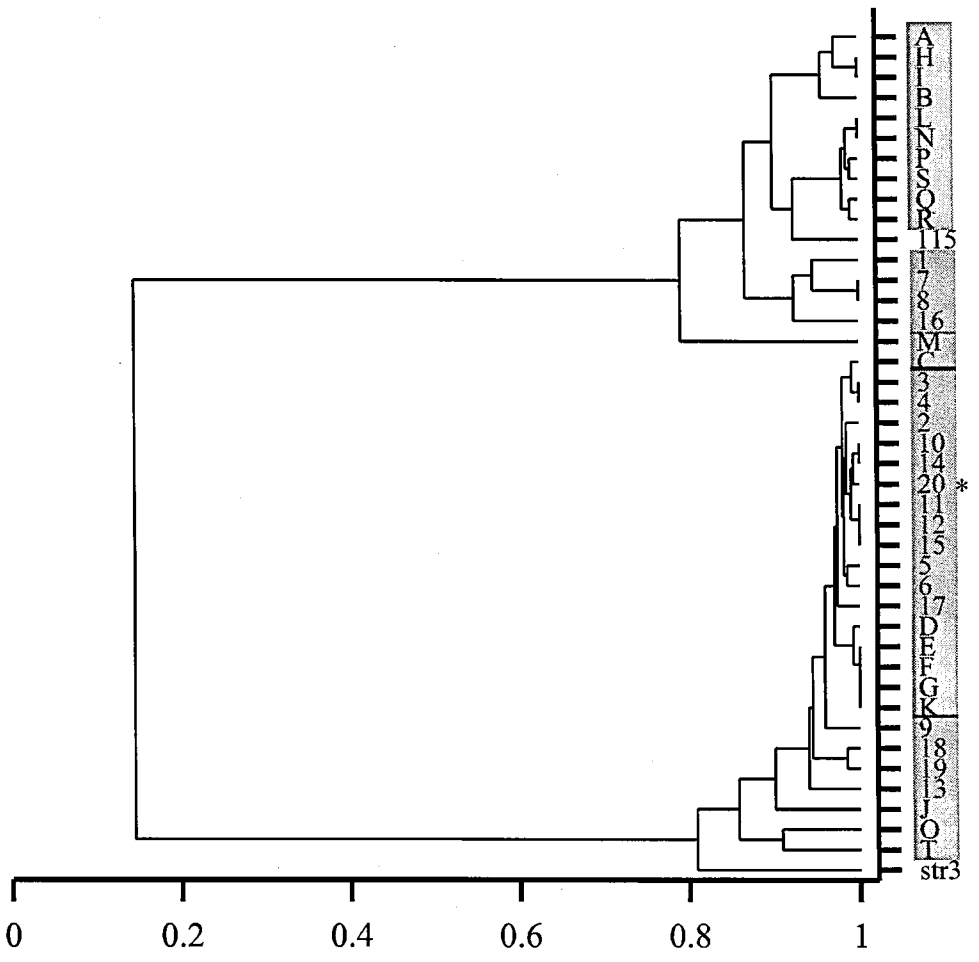

Figure 3.3a: Dendrogram derived from analysis of all AFLP fingerprints of 40 mono-conidial isolates and their 'parents', *V. dahliae* 115 and *V. albo-atrum* STR3. Numbered isolates are derived from microsclerotium 12■ , and letters from 11 ■. *Isolate 12.20 showed a novel combination of *nit* mutant/ITS phenotypes.

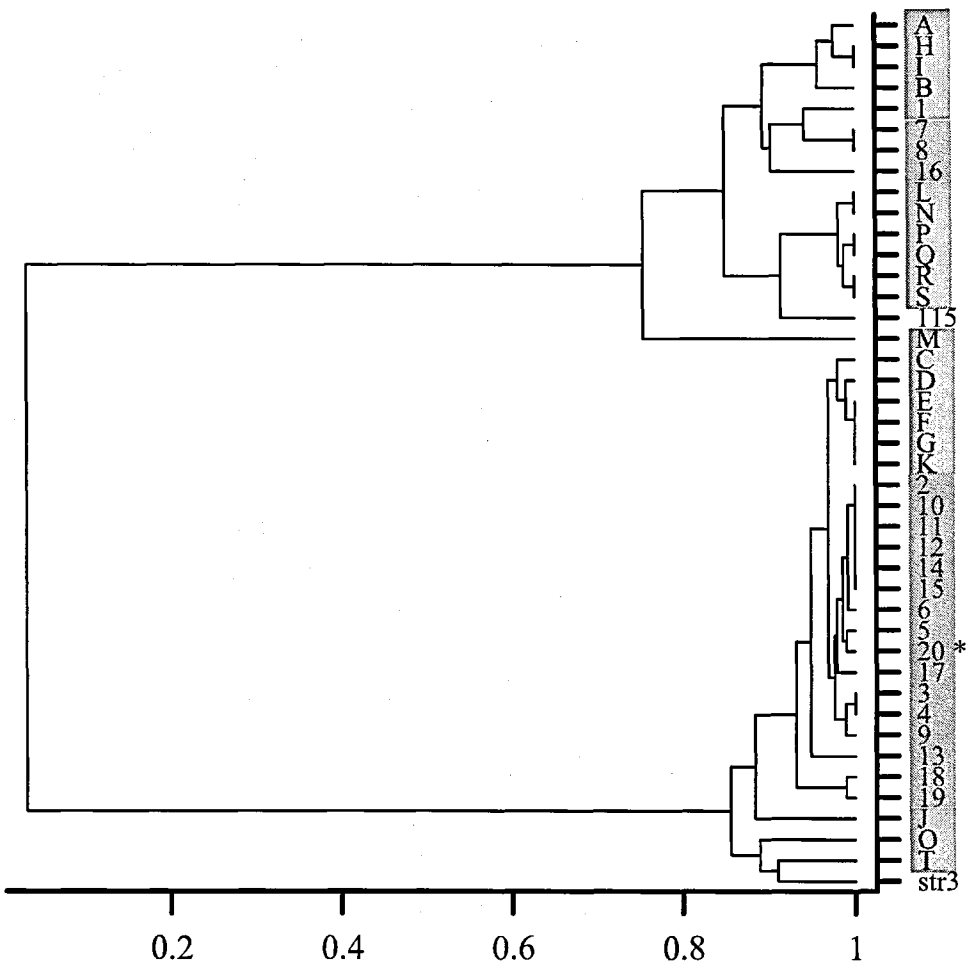

Figure 3.3b. Dendrogram from analysis of AFLP fingerprint derived from 40 mono-conidial isolates and their 'parents' based on a score of 0 or 1 in either 'parents' *V. dahliae* 115 and *V. albo-atrum* STR3. Numbered isolates are derived from microsclerotium 12□, and letters from 11□. *Isolate 12.20 showed a novel combination of *nit* mutant/ITS phenotypes.

V. albo-atrum AFLP clade													
Primer combination	651/655	651/655	651/655	651/658	652/658	651/655	651/655	651/655	651/655	651/655	652/658	652/658	653/655
11c	1	1	1	1	1	0	0	0	0	0	1	0	1
11d	1	1	1	1	1	0	0	0	0	0	1	0	1
11e	1	1	1	*	1	0	0	0	0	0	1	0	1
11f	1	1	1	1	1	0	0	0	0	0	1	0	1
11g	1	1	1	1	1	0	0	0	0	0	1	0	1
11j	1	1	1	1	1	0	0	0	0	0	1	0	1
11k	1	1	1	1	1	0	0	0	0	0	1	0	1
11o	1	0	0	1	1	1	1	1	1	1	1	0	0
11t	0	0	0	1	1	1	1	1	1	1	1	0	0
12.02	1	1	1	*	*	0	0	0	0	0	*	*	1
12.03	1	1	1	*	1	0	0	0	0	0	1	0	1
12.04	1	1	1	*	1	0	0	0	0	0	1	0	1
12.05	1	1	1	0	1	0	0	0	0	0	1	0	1
12.06	1	1	1	0	1	0	0	0	0	0	1	0	1
12.09	1	1	1	0	1	0	0	0	0	0	1	0	1
12.10	1	1	1	1	1	0	0	0	0	0	1	0	1
12.11	1	1	1	1	1	0	0	0	0	0	1	0	1
12.12.	1	1	1	1	1	0	0	0	0	0	1	0	1
12.13	1	1	1	1	1	0	0	0	0	0	1	0	1
12.14	1	1	1	1	1	0	0	0	0	0	1	0	1
12.15	1	1	1	1	1	0	0	0	0	0	1	0	1
12.17	1	1	1	1	1	0	0	0	0	0	1	0	1
12.18	1	1	1	1	1	0	0	0	0	0	1	0	1
12.19	1	1	1	1	1	0	0	0	0	0	1	0	1
12.20	1	1	1	1	1	0	0	0	0	0	1	0	1
STR3	**0**	**0**	**0**	**0**	**0**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**
V. dahliae AFLP clade													
Primer combination	651/655	651/655	651/655	651/658	652/658	651.655	651.655	651/655	651/655	651/655	652/658	652/658	653/655
11a	0	0	0	1	1	1	1	1	1	1	1	1	0
11b	0	0	0	1	1	1	1	1	1	1	1	1	0
11h	0	0	0	1	1	1	1	1	1	1	1	1	0
11i	0	0	0	1	1	1	1	1	1	1	1	1	0
11l	0	1	1	1	1	0	0	0	0	0	1	1	0
11n	0	1	1	1	1	0	0	0	0	0	1	1	0
11p	0	1	1	1	1	0	0	0	0	0	1	1	1
11q	0	1	1	1	1	0	0	0	0	0	1	1	1
11r	0	1	1	1	1	0	0	0	0	0	1	1	1
11s	0	1	1	1	1	0	0	0	0	0	1	1	1
12.01	0	0	0	*	*	1	1	1	1	1	*	*	0
12.07	0	0	0	1	1	1	1	1	1	1	1	1	0
12.08	0	0	0	1	1	1	1	1	1	1	1	1	0
12.16	0	0	0	0	1	1	1	1	1	1	1	1	0
11m	0	0	0	1	1	0	0	0	0	0	1	1	0
115	**1**	**1**	**1**	**1**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**

Table 3.1. Summary table showing putative recombination at some loci between the 'parental' *V. dahliae* 115 isolates and *V. albo-atrum* STR3 genomes in the progeny isolates. Shaded indicates *V. dahliae* derived polymorphism shaded is *V. albo-atrum* derived polymorphism. * indicates missing data.

Monoconidial isolate	rRNA ITS	*nit* phenotype	AFLP clade
115	***V.dahliae***	***nit*M**	***V. dahliae***
STR3	***V.albo-atrum***	***nit*1**	***V. albo-atrum***
11a	*V. dahliae*	*nit*M	*V. dahliae*
11b	*V. dahliae*	*nit*M	*V. dahliae*
11c	*V. albo-atrum*	*nit*1	*V. albo-atrum*
11d	*V. albo-atrum*	*nit*1	*V. albo-atrum*
11e	*V. albo-atrum*	*nit*1	*V. albo-atrum*
11f	*V. albo-atrum*	*nit*1	*V. albo-atrum*
11g	*V. albo-atrum*	*nit*1	*V. albo-atrum*
11h	*V. dahliae*	*nit*M	*V. dahliae*
11i	*V. dahliae*	*nit*M	*V. dahliae*
11j	*V. albo-atrum*	*nit*1	*V. albo-atrum*
11k	*V. albo-atrum*	*nit*1	*V. albo-atrum*
11l	*V. dahliae*	*nit*M	*V. dahliae*
11m	*V. dahliae*	*nit*M	*V. dahliae*
11n	*V. dahliae*	*nit*M	*V. dahliae*
11o	*V. albo-atrum*	*nit*1	*V. albo-atrum*
11p	*V. dahliae*	*nit*M	*V. dahliae*
11q	*V. dahliae*	*nit*M	*V. dahliae*
11r	*V. dahliae*	*nit*M	*V. dahliae*
11s	*V. dahliae*	*nit*M	*V. dahliae*
11t	*V. albo-atrum*	*nit*1	*V. albo-atrum*
12.01	*V. dahliae*	*nit*M	*V. dahliae*
12.02	*V. albo-atrum*	*nit*1	*V. albo-atrum*
12.03	*V. albo-atrum*	*nit*1	*V. albo-atrum*
12.04	*V. albo-atrum*	*nit*1	*V. albo-atrum*
12.05	*V. albo-atrum*	*nit*1	*V. albo-atrum*
12.06	*V. albo-atrum*	*nit*1	*V. albo-atrum*
12.07	*V. dahliae*	*nit*M	*V. dahliae*
12.08	*V. dahliae*	*nit*M	*V. dahliae*
12.09	*V. albo-atrum*	*nit*1	*V. albo-atrum*
12.10	*V. albo-atrum*	*nit*1	*V. albo-atrum*
12.11	*V. albo-atrum*	*nit*1	*V. albo-atrum*
12.12	*V. albo-atrum*	*nit*1	*V. albo-atrum*
12.13	*V. albo-atrum*	*nit*1	*V. albo-atrum*
12.14	*V. albo-atrum*	*nit*1	*V. albo-atrum*
12.15	*V. albo-atrum*	*nit*1	*V. albo-atrum*

12.16	*V. dahliae*	*nit*M	*V. dahliae*
12.17	*V. albo-atrum*	*nit*1	*V. albo-atrum*
12.18	*V. albo-atrum*	*nit*1	*V. albo-atrum*
12.19	*V. albo-atrum*	*nit*1	*V. albo-atrum*
12.20	***V. dahliae***	***nit*1**	***V. albo-atrum***

Table 3.2. Summary Table of results from interspecific *nit* mutant pairings. In bold shows the novel combination of *nit* phenotype with rRNA gene ITS.

3.3.2.1 Protoplast generation and purification

Protoplasts were obtained from all isolates tested, using the protocol of Okoli (1992), with the Lysing Enzyme from *Trichoderma* sp. and Driselase in 1.2M $MgSO_4$ incubated for 150-180 minutes at 30°C at 80 rpm. Protoplasts were purified by creating an interface between the 1.2M $MgSO_4$ digest mixture overlaid with 600mM sorbitol, 100mM tris.HCl pH 7.0. During centrifugation, protoplasts collected at the interface and were removed and further washed. Post-purification protoplast concentrations were in the range 3.8×10^6 – 1.9×10^7 per ml. The proportion of undigested spores found in these purified protoplast suspensions was between 0-5%.

Percoll proved not to be as an effective method of purification as that of Okoli (1992). The presence of Percoll, prepared with 800mM sorbitol, 10mM trisHCl pH7.0, in the digest mixture during the incubation resulted in 0 - 4.4×10^4 protoplasts per ml for the isolates tested. When Percoll prepared with 800mM sorbitol, 10mM trisHCL was added to the digest mixture post-incubation protoplasts were produced at a final concentration of $2.2 \times 10^5 - 1 \times 10^6$ per ml but within this final purified suspension $1 \times 10^5 - 1 \times 10^6$ per ml spores were found.

Percoll added to the digest mixture resulted in undigested mycelia, spores and other detritus collecting with protoplasts at the interface. Percoll added post-incubation to the digest mixture caused lysis of the protoplasts.

The modified protocol of Fieuw and Willenbrink (1991) where protoplast purification was based on flotation on 15% and 25% Percoll with 600mM sorbitol resulted in undigested

mycelia, spores and detritus collecting at the meniscus with protoplasts. Following on from these results, reduced concentrations of Percoll (5 – 12.5%) with 600mM sorbitol were tested. It was observed that for all concentrations of Percoll tested the relatively smaller protoplasts collected with smaller debris and spores at the flotation stages whereas the large protoplasts collected with undigested mycelia and swollen spores in the pellet. It was also noted that swollen spores were present during the mycelial digest.

3.3.2.2 Single carbon substrate utilisation profiles of three different *Verticillium* isolates

Carbon substrate utilisation profiles of three isolates (12087, STR3 and VdII) were assessed using the API 50 CH strip system. The profile of each isolate was read and scored after 8 days incubation in the dark at 20 - 25°C. Growth was scored in relation to the control tube and scored on a 6 point scale. (Table 3.3)

STR3 was able to assimilate more substrates (21 substrates at score 2 or 3) than either 12087 (two substrates at score 2 or 3) and VdII (four at score 2 or 3). All four of the substrates utilised well by VdII were also utilised well by STR3, as were both of the substrates used well by 12087. Thirteen of the substrates used well by STR3 were used very poorly (-2 to 0) by VdII.

Substrate	12078	STR3	VdII
Control	0	0	0
Glycerol	-1	1	0
Erythritol	0	1	0
D-arabinose	0	1	0
L-arabinose	1	1	0
Ribose	1	1	0
D-Xylose	1	1	0
L-Xylose	0	1	-1
Adonitol	0	1	-1
β Methyl-xyloside	0	1	0
Galactose	1	1	-1
D-Glucose	1	1	0
D-Fructose	1	0	0
D-Mannose	1	0	0
L-Sorbose	-1	0	-1
Rhamnose	1	0	0
Dulcitol	0	1	-1
Inositol	0	0	-1
Mannitol	0	1	-1
Sorbitol	0	1	-1
α Methyl-D-mannoside	0	2	0
α Methyl-D-Glucoside	1	2	0
N Acetyl glucosamine	1	2	0
Amygdaline	0	2	0
Arbutine	No result	1	0
Esculine	No result	No result	1
Salicine	1	2	2
Cellobiose	1	2	1
Maltose	1	2	0
Lactose	1	2	0
Melibiose	1	2	1
Saccharose	0	2	0
Trehalose	1	2	1
Inuline	2	3	3
Melezitose	1	1	1
D-Raffinose	1	1	1
Amidon	1	2	2
Glycogen	2	3	3

Xylitol	0	2	0
β Gentibiose	1	1	1
D-Turanose	0	1	1
D-Lyxose	-2	2	-1
D-Tagatose	-1	2	0
D-Fucose	-2	2	-1
L-Fucose	1	1	1
D-Arabitol	1	2	1
L-Arabitol	1	1	0
Gluconate	1	1	1
2 keto-gluconate	0	2	0
5 keto-gluconate	0	2	0

Table 3.3. Carbon substrate profiles of three *Verticillium* isolates (12078, STR3, and VdII). 0=control or same observed growth as control; -2=no growth; -1=some evidence of fine mycelial growth; +1=mycelial growth and/or production of some resting structures; +2=thick mycelial growth and some production of resting structures; +3=thick mycelial growth and profuse resting structure production.

3.4 Discussion

3.4.1 Interspecific *nit* mutant complementation

PCR amplification of cultures 11 and 12 detected the presence of both *V. dahliae* and *V. albo-atrum* rRNA ITS types. It is possible that there were cells present from each of the 'parent' within the single microsclerotium or it may have been that through the transfer of the microsclerotium, mycelia or conidia from both parental *nit* mutant isolates may also be transferred. The preparation of mono-conidial cultures from 11 and 12 should have ensured that only one ITS type was present in the 'offspring' cultures, if the monoconidial isolate was haploid and if the conidia were mononucleate. The presence of both ITS types detected by PCR amplification in single mono-conidial isolates might be because conidia are heteronucleate (*e.g.* two or more haploid nuclei that are genetically different) or mononucleate with fused amphihaploid nuclei (*e.g.* two genomes of unequal chromosome number existing in a fused nucleus). In a few natural amphihaploid isolates both a major and minor ITS types were found (Collins *et al.*, 2003) (see 5.1.2.1 for an explanation of major and minor rRNA ITS types) and it is also possible that both parental rRNA arrays were present in haploid or aneuploid progeny.

The apparent presence of both *V. dahliae* and *V. albo-atrum* ITS types initially in cultures 11c, 11r and 11s may have been due to the latter two possibilities but, the repeat squash blot and PCR amplification of the ITS, to some extent, discounts this. Although, the results do not rule out that heterokaryons or even true hybrids with fused nuclei between the two parental isolates may have formed and have subsequently broken down in successive generations.

Analysis of the *nit* phenotype of the twenty monoconidial isolates from each of cultures 11 and 12 showed that one monoconidial isolate (12.20) had a novel combination of *nit* phenotype with rRNA ITS type. Taken at face value this result indicates that some form of genetic exchange occurred either within the heterokaryon or a true hybrid with true fused nuclei was formed. However, as only a single isolate is involved this requires independent confirmation.

AFLP analysis was used to look at the variation within the forty mono-conidial isolates, their relationship to the 'parental' isolates, and for possible recombination between the two 'parental' genomes in the mono-conidial isolates. AFLP analysis clearly divided the 40 mono-conidial isolates tested into two groups that separated the 'parental' *V. dahliae,* and those isolates that were akin to it, from the *V. albo-atrum nit* mutant and like isolates respectively. This for the main corroborated the findings from analysis of the rRNA ITS type and that of the *nit* phenotype. Through AFLP it was shown that none of the offspring were genetically identical to the parent. Although this indicates a degree of genetic exchange, the presence of two distinct clades (each like one or the other parent isolates), suggest that the genetic exchange was limited and/or that only *V. albo-atrum* or *V. dahliae*-like combinations survive.

Electrophoretic karyotyping undertaken in Collins *et al.* (2003) implied that there were 6 chromosomes in haploid *V. dahliae* and 7 in *V. albo-atrum*. This inequality of numbers of chromosomes was not reflected in a difference of implied genome size (*V. dahliae* genome 26.6-29.1 Mbp; *V. albo-atrum* genome 28.1-29.9 Mbp), it however may account for the apparent instability of the presumed hybrids in this study.

It is also apparent from this work that the recombination observed in 11 and 12 were different due to the different clustering observed. Isolates derived from 11, for the main part, only group with other isolates from 11 and the same is true for isolates from 12 (Figure 3.3a, b) although some isolates (notably 11m in one clade and 11j, 11o, and 11t in the second) seem more divergent.

A study of vegetative compatibility and heterokaryon stability in *Fusarium oxysporum* f.sp. *radicis lycopersici* analysed the stability of prototrophic heterokaryons between complementary *nit* mutants through mycelial mass transfer and conidial analysis (Di Primo *et al.*, 2001). The authors concluded that heterokaryons do not survive well through conidiation, ergo, if a similar system operates in plant pathogenic *Verticillium*, two complete genomes would not be maintained through the preparation of monoconidial isolates from heterokaryons. However, if any true hybrids arising through nuclear fusion are stable these should survive the selection of monoconidial isolates.

If irregular microsclerotia are a result of two genomes existing independently within a heterokaryon, the haploid progeny should be considered genetically the same as one or other of the parental isolates. The fact that none of the monoconidial isolates demonstrated prototrophic growth on MM and that AFLP analysis showed apparent little recombination between the parental genomes in the progeny, suggest that no true hybrids with fused nuclei were formed. However, as none of the progeny were genetically identical, according to AFLP, to either 'parents' therefore some genetic exchange must have occurred. This complementation of interspecific *nit* mutants was carried out as a preliminary experiment to establish whether it was worth going ahead with fusion to do the experiment more

rigorously. From this preliminary work it seems that it was promising enough to carry on, but this was not done because of the doubts over the identity of the parents of the natural amphihaploids.

3.4.2 Protoplast generation and purification

Before protoplast fusion, it is necessary that the protoplast suspensions are free from spores, mycelia and detritus. This is important, as in this case no artificial method of selection was to be employed in either the generation of auxotrophic mutants or through transformation with a selectable marker, and excessive numbers of spores and mycelia would mask any potential regeneration of protoplasts.

The protocol of Okoli (1992) provided good levels of efficacy in separating protoplasts from spores for the isolates tested, thus this method of protoplast generation would be suitable for PEG fusion techniques. It was, however, an intention to use electrofusion techniques in producing a hybrid and for this purpose the protocol of Okoli (1992) would be unsuitable due to the high content of ionic material in the final preparation. The presence of $MgSO_4$ in the enzyme mixture does not lend itself to electrofusion due to its high conductivity and it is necessary to utilise a protoplast generation procedure that does not rely upon $MgSO_4$ or ionic osmoticants to guarantee no carry-over into the electrofusion process. Percoll was tested for its effectiveness in producing a mixture with sorbitol of sufficient density so that 0.6M sorbitol may be overlaid onto the digest mixture, with the intention that post-centrifugation a band of protoplasts would form at the interface of the two liquids. Percoll was chosen for test here as it is silica based and both osmotically and ionically inert. It was assumed that spores, undigested mycelia and detritus would pellet or

be at a lower position within the tube. The methods of this type tested have as yet not been successful.

For 50% Percoll in 800mM sorbitol, 10mM trisHCl solution added to the enzyme digest, the mixture post-digest was too dense for any separation of protoplasts from spores, undigested mycelia and general detritus to occur. Results from the modified method of Fieuw and Willenbrink (1991) using 15% and 25% Percoll were ambiguous and would require further development as the presence of swollen spores in all phases of the sorbitol/Percoll solution indicates that it is important to inhibit any swelling the spores may undergo during the enzyme digest. This important as the presence of swollen spores hinders the purification of protoplasts in this manner.

3.4.3 Single carbon substrate utilisation profiles

To create artificial hybrids, without using auxotrophic mutants in the progenitor isolates, meant that there would be difficulties in their selection and that it would be necessary to rely on morphological characteristics normally associated with crucifer isolates from the field such as long spores. The API strips were utilised to try to find differential carbon substrate utilisations between species with as a possible means of selection for hybrids. This of course assumes that artificial hybrids would have properties similar to those of field hybrid isolates and that their metabolic profiles would be similar if not the same. Once selective substrates had been identified for the respective isolates, a hybrid could be selected for by switching from substrate or a combination of substrates to an alternate combination of substrates. After both stages, hybrids progeny that would be able to utilise

all substrates at each stage would be selected for as they could potentially metabolise all substrates used.

For the three isolates tested there was some observed growth in the control tube however, this might be due to the possible carry over of carbohydrates from the spore suspension prepared by washing the plate with sterile distilled water. In any further experiments, it would probably be helpful to wash the spores after collection either in MM without sucrose or sterile distilled water prior to inoculation into the strip.

For isolate 12087 arbutine and esculine produced no result. This was due to the production of dark pigment thus obscuring any mycelial growth that may be present in the tubule. STR3 produced no result for the substrate esculine, again because of the production of dark pigment obscuring any possible mycelial growth.

Isolate 12087 had no substrate that was given a score of +3, and only two substrates had score of +2 (inuline and glycogen). There were 24 substrates that produced a core of +1 and 16 produced growth equivalent to that of the control tube. It was found however that a four substrates seemed to inhibit the growth of 12087 with scores of –1 (glycerol and d-tagatose) and –2 (d-lyxose and d-fucose) respectively.

For STR3, all substrates (except esculine) produced equivalent growth to or better than the control tube. Two substrates gave scores of +3 (inuline and glycogen). A score of +2 was given to 10 substrates in STR3, and 22 substrates had a score of +1. There were 5 substrates that produced growth equivalent to the control tube and were thus scored 0.

The *Verticillium* crucifer isolate, VdII, like STR3 had two substrates that gave scores of +3 and like STR3 these were inuline and glycogen. Two substrates gave scores of +2 on the arbitrary scale (amidon and salicine), 10 substrates had scores of +1, and 24 scored as that of the control tube 0. It was shown that 10 substrates inhibited the growth of VdII with a score of –1.

It was surprising that VdII (the natural hybrid) was able to use fewer substrates than either 12087 or STR3. This was further early evidence that the amphihaploids did not arise from 'parents' similar to these isolates or, possibly less likely, that there is repression in the natural hybrid. As the amphihaploid uses few substrates it was not possible to find a combination that would allow either direct selection for a novel hybrid or sequential selection by for example by using a substrate on which *V. albo-atrum* and the artificial hybrid grows (with haploid *V. dahliae* not growing), then moving to a substrate on which *V. dahliae* and the artificial hybrid could grow but not *V. albo-atrum*. This indicates that this system alone could not provide the means to identify any possible selective substrates that can select for an artificial hybrid.

The study of sole carbon substrate utilisation profiles alone did not provide potential means of non-mutagenic selection for novel *V. dahliae* x *V. albo-atrum* hybrids. However, a combination of carbon and nitrogen sources might be greater use. Further analysis using API strips (or a similar product) for sole carbon substrate utilisation along with nitrogen sources might prove effective.

3.4.4 Concluding Remarks

Removing the barriers of heterokaryon incompatibility, via microinjection or protoplast fusion allows for extensive interaction between the two species (probably with more ease than with which it could occur in nature) and also permits a reliable artificial system for comprehensive genetic and molecular analysis. It was this premise that supported the aims behind this chapter, and initially this PhD project. It was intended to create artificial interspecific hybrids using protoplast fusion without inducing auxotrophic mutants between *V. dahliae* and *V. albo-atrum* and analyse isolates that resulted. Not using auxotrophs would make selecting for hybrids difficult, however, it was thought desirable to do so as it would not be certain if mutaginisation had caused changes in pathogenicity genes. Although pathogenicity could be assessed, it would not be clear whether deleterious mutations had occurred in the genes associated with the novel pathogenicity associated with crucifers. As a consequence of difficulties in selection of hybrids, it would be necessary to select using morphological properties such as long-spores using flow cytometry.

As discussed previously where extensive analyses of a wide range of cruciferous isolates alongside haploid isolates of *V. dahliae* and *V. albo-atrum* by a range of molecular methods demonstrated that at least one of the parents in the supposed hybridisation events appears to be a new, as yet unidentified species. The isolates involved in the hybridisation events may be of ancient origin and are no longer in existence (although at least one would still have been quite distinct molecularly from modern isolates (See Chapter 6).

However what had become clear at this stage was that for the immediate purposes of this project the construction of artificial amphihaploids using known isolates of *V. albo-atrum* and *V. dahliae* may well not deliver the originally intended increased understanding of pathogenicity. This is primarily because if any novel hybrids generated, and this might require extensive work to determine, did not possess pathogenicity for crucifers, it could be argued that this was simply because the 'wrong' parent had been used. Therefore it was decided not to proceed with this approach but to focus much more strongly on characterising the natural hybrids and try to look for the 'true' parent of natural hybrids by molecularly characterising any unusual isolates that could be gathered from across the world.

4 PATHOGENICITY AND *VERTICILLIUM* ISOLATES FROM CRUCIFERS

4.1 Introduction

4.1.1 Host-range specificity in *V. dahliae* and *V. albo-atrum*

In general the plant pathogenic *Verticillium* species have broad host ranges and many isolates reflect this. However, varying degrees of host-specificity and host-adaptation have been reported.

Within *V. dahliae* haploid isolates, those from peppermint have been reported to form a separate physiological race with a degree of host specialisation (Nelson, 1950). Isolates from sweet pepper are reportedly host-adapted (Kendrick and Middleton, 1959) as are those from bell pepper (Bhat and Subbarao, 1999). In the work of Bhat and Subbarao (1999) isolates of *V. dahliae* from bell pepper, cotton, eggplant and mint all exhibited a degree of host specificity. Furthermore, bell pepper was resistant to all *Verticillium* isolates tested except those from bell pepper and eggplant.

Similar results have been found in studies carried out as part of an EU project (QLRT-1999-1523) (Personal communication, Dr D. J. Barbara – Warwick HRI). From this work it is apparent that some hosts can be used to differentiate between isolates, and that host-specificity exists in some isolates of *V. dahliae*. For example in Figure 4.1, it can be clearly observed in pathogenicity testing on two hosts that *V. dahliae* fell into two populations, those isolates that are more virulent on potato than melon and *vice versa.* In Figure 4.2 there is continuous variation in pathogenicity on resistant eggplant but on olive isolates are

either pathogenic or not with no variation in between. In addition, those isolates that did produce any symptoms on cotton were found to be defoliating isolates.

4.1.2 Host-specificity of *Verticillium* isolates from crucifers

Apparent host specificity towards a cruciferous crop was first recorded in 1957 by Ivor Isaac (Isaac, 1957). A microsclerotia forming species, *V. dahliae*, had been causing wilt in Brussel sprout plants on a farm near Evesham, Worcestershire, UK. This was not the first incidence of *Verticillium* wilt in Brussel sprout as Snyder *et al.* (1950) reported a wilt in this crop in the USA induced by a fungus that they termed *V. albo-atrum*. At that time, there was some confusion with regard to species identity of *V. dahliae* and *V. albo-atrum* isolates, as some groups considered that both microsclerotial and dark resting mycelial forms of *Verticillium* were the same species *i.e. V. albo-atrum*. Thus, it is possible that the isolates causing *Verticillium* wilt of sprouts in both countries may in fact have been similar.

Snyder *et al.* (1950) reported that their isolate from Brussel sprouts had a narrow range of pathogenicity since it had no effect on tomato, a plant usually susceptible to infection by *V. dahliae*. In studies on the UK Brussel sprout isolate, its pathogenicity on a range of host crops was compared with two other isolates (from antirrhinum and phlox). The data showed that the isolate from Brussel sprouts was a distinct strain with a more restricted pathogenicity than shown by either of the antirrhinum or phlox isolates. Two cruciferous crops were included in the test, broccoli and cauliflower, but these showed no wilt when infected by any of the three isolates. Antirrhinum and phlox plants showed 100% and 90% wilt symptoms for their respective isolates, however, no Brussel sprout plants were

included in the tests (Isaac, 1957). The author concluded that his results agreed, to a certain extent, with that of Snyder *et al.* (1950). However, it is to be highlighted that in his tomato and potato plants, although infection was established in some plants inoculated with the Brussel sprout isolate, their development was much slower and the symptoms far less severe than that induced by the other two isolates. Isaac (1957) concludes by advising the farmer to grow less susceptible crops, such as cauliflower and broccoli, after Brussel sprouts.

Later studies (Subbarao *et al.*, 1995) showed that isolates from cauliflower and cabbage caused wilt on both hosts and similarly a strawberry isolate could cause wilt on cauliflower plants and *vice versa*. In this study, the authors conclude that host specificity was not observed, even in isolates from within the cruciferous crops. Isolates from cauliflower reduced height in inoculated broccoli and Brussel sprout plants, and exhibited a significantly higher severity index than uninoculated plants. Although the cauliflower isolates could infect Brussel sprouts and broccoli, they were only weakly pathogenic. It should be noted that *Verticillium* wilt has never been observed in commercial broccoli fields in Salinas Valley, CA, even when broccoli is planted in soils rich in microsclerotia (Koike *et al.*, 1994).

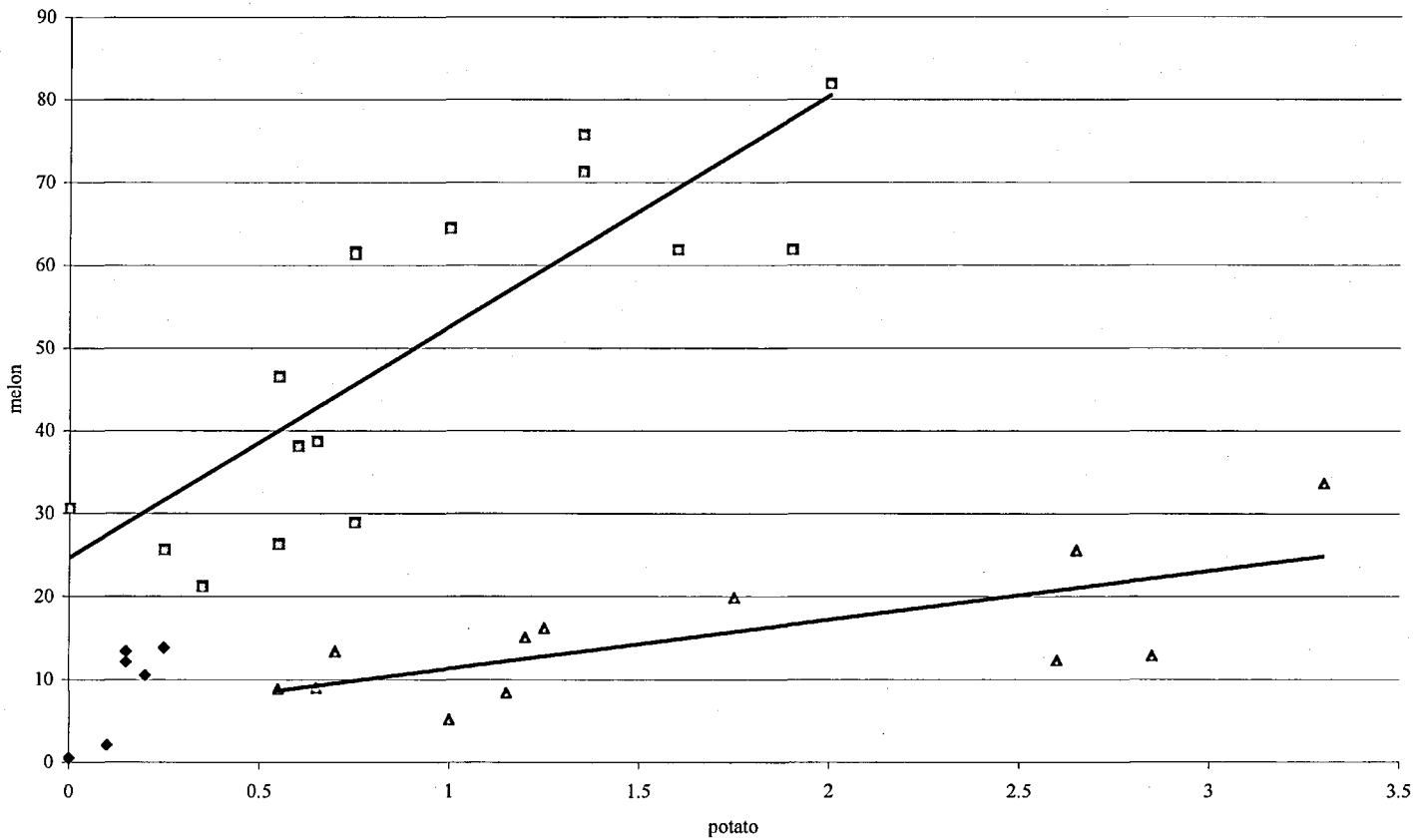

Figure 4.1. Potato vs. Melon pathogenicity data, courtesy Dr D. J. Barbara, EU project QLRT-1999-1523 (*Verticillium* in trees). Δ Potato-type specific isolates □ Melon-type specific isolates ◆ Isolate not easily decided.

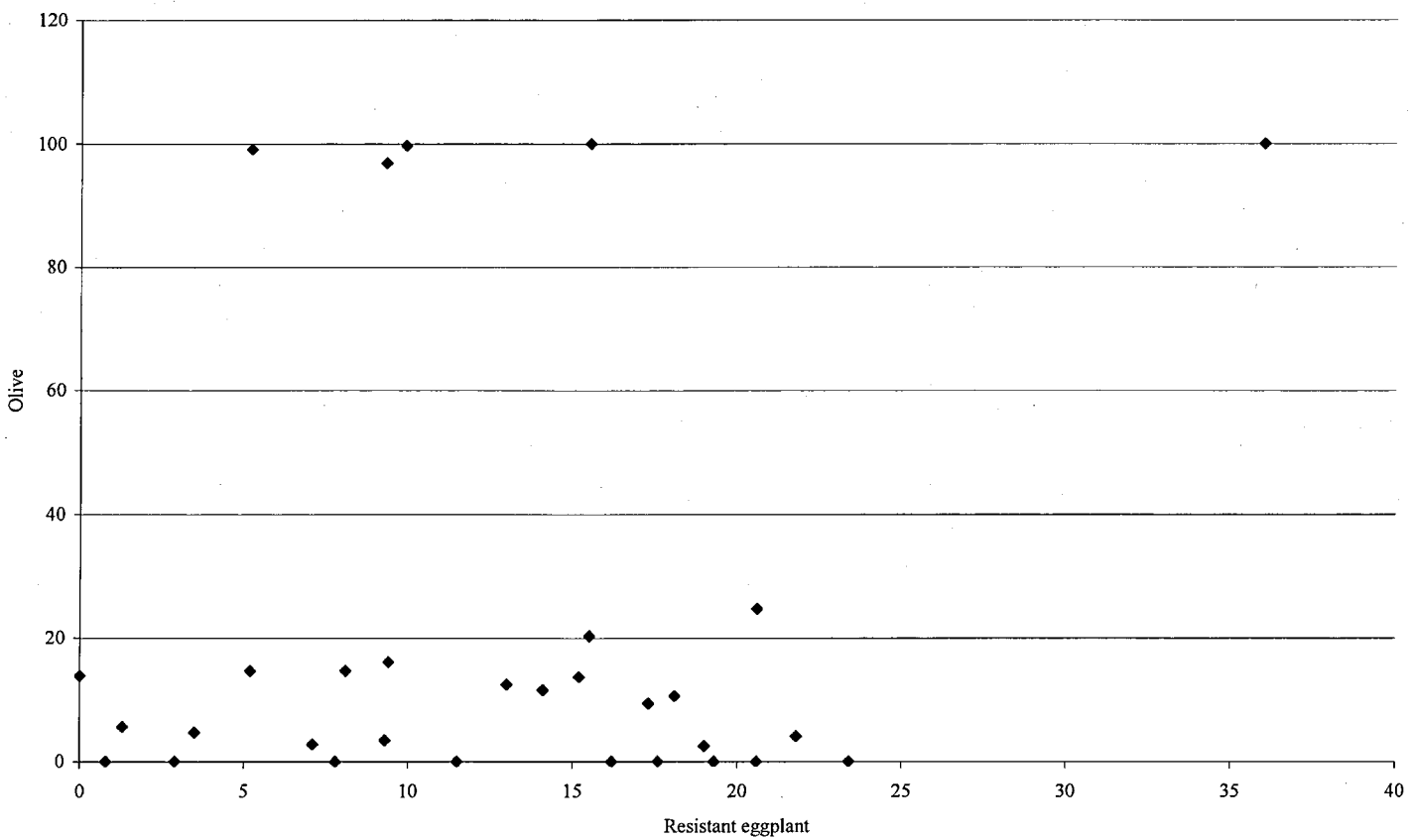

Figure 4.2. Resistant Eggplant vs. Olive pathogenicity data. Data courtesy of Dr D. J. Barbara EU project QLRT-1999-1523 (*Verticillium* in trees).

Subbarao *et al.* (1995) also found that isolates from certain non-cruciferous crops were as virulent on cauliflower as cauliflower isolates, and the same cauliflower isolates were also virulent on non-cruciferous crops. Whilst the *V. dahliae* isolates from non-cruciferous crops were pathogenic to cauliflower and *vice versa*, there were differences in their virulence (Figure 4.3). Later studies included an isolate of *V. albo-atrum* from Lucerne that was found to be equally as virulent as crucifer isolates on cruciferous crops as it was to alfalfa, and other non-cruciferous crops (Bhat and Subbarao, 1999).

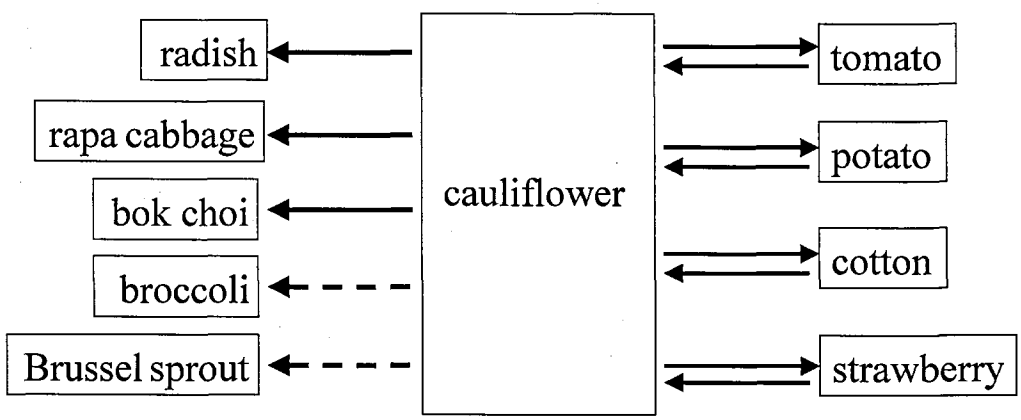

Figure 4.3. Summary of the pathogenicity tests of different *V. dahliae* isolates on different hosts. Arrows pointing both ways indicate that the isolate originating from each was tested on the other. Arrows in one direction means that only cauliflower isolates were inoculated. Dashed arrows indicate that the isolates were weakly pathogenic on crops. Adapted from Subbarao *et al.* (1995).

Similar studies by Chang and Eastburn (1994) evaluated the pathogenicity of horseradish isolates on herbaceous plant species. China aster, eggplant, horseradish, potato and sunflower showed symptoms typical of *Verticillium* wilt, such as leaf chlorosis, necrosis and vascular discolouration. Pepper, tomato and watermelon were asymptomatic hosts, since plants were colonised by the pathogen but lacked symptoms.

Studies regarding host specificity relating crucifer isolates to haploid *V. dahliae* isolates were undertaken by Horiuchi *et al.* (1990) and differentiated haploid *V. dahliae* and crucifer isolates into 4 groups. Group A, eggplant strain, comprised isolates that were pathogenic to eggplant and turnip. Group B, tomato strain, isolates that were pathogenic to eggplant, tomato and turnip. Group C, sweet pepper strain, isolates were pathogenic to eggplant, sweet pepper and turnip. Finally, group D, isolates that were pathogenic to turnip (or crucifers alone) alone. All isolates in group D were taken from cruciferous hosts, whereas those of A, B, and C were from plants of various hosts including Cruciferae. It was found that there was no evidence for the alteration of pathogenic ability in any isolate group (Horiuchi *et al.*, 1990).

Horiuchi *et al.* (1990) also identified certain morphological characteristics associated with group D isolates. Within A, B, and C no clear differences were found with regard to morphology but group D isolates were noted to have longer conidia and distinctive conidiophores and microsclerotia. Finally, group D isolates were directly compared to known isolates from crucifers and tested enzymatically and pathogenically. It was found that the known isolates from crucifers were closely allied to group D isolates and a strong relationship between the two was inferred.

Studies by Karapapa *et al.* (1997) tested *Verticillium* from crucifers (the majority coming from oilseed rape with a limited number of isolates from crucifers in Japan) on oilseed rape plants. The majority of crucifer isolates were pathogenic to oilseed rape except for Starks' horseradish isolate and a German oilseed rape isolates G19 (MD73 as designated in Messner *et al.*, 1996) that were only weakly pathogenic. Zeise and Buchmuller (1997) tested a typical oilseed rape isolate against six *Brassica* species. Severe wilt was evident on *B. campestris*, *B. nigra*, and *B. napus* and although less severe symptoms were evident in *B. juncea*, yield losses were high. The most resistant species, showing no yield losses, were *B. carinata* and *B. oleracea*. Steventon *et al.* (2001) showed that oilseed rape isolates were pathogenic to *Arabidopsis thaliana* and another study showed that such isolates they were non-pathogenic to non-cruciferous crops including potato, faba bean, lupin, pea, tomato and potato (Zeise and von Tiedemann, 2002b).

Therefore, overall there is some confusion as to the degree of host specificity shown by *Verticillium* isolates from crucifers, with isolates from, for example, cauliflower apparently being able to cause disease on non-cruciferous hosts but those from oilseed rape not doing so. All these studies involved artificial inoculation, by dipping cut roots, and it is not clear how much differences in technique are responsible for the varying results. Nor is one host common to these studies and varying degrees of resistance by various crucifers may be involved *e.g. Arabidopsis* seems highly susceptible to all isolates of both *V. albo-atrum* and *V. dahliae*.

It is to be explained, however, that host specificity does seem to occur in the field as the overwhelming majority of isolates from naturally infected crucifers appear to be long-spored.

4.1.3 Aims and Objectives

Three questions will be addressed through this study.

- Do amphihaploid isolates of *Verticillium* from crucifers differ from haploid *Verticillium* isolates in their pathogenicity towards a single cruciferous crop (oilseed rape)?
- Are isolates from crucifers host-specific?
- If an artificial interspecific hybrid was generated would it have been possible to identify it on the grounds of novel pathogenicity towards a cruciferous crop?

The first question will be tested using a wide range isolates from *V. dahliae*, *V. albo-atrum*, *Verticillium* isolates from crucifers and related species against a single cruciferous crop, oilseed rape, using an invasive method of inoculation, the cut root method.

The second question will be tested using a smaller range of isolates of mainly *Verticillium* isolates from crucifers, *V. dahliae* and *V. albo-atrum* against two crops, oilseed rape and horseradish using infested soil with no deliberate root damage. Through the answers to the first two questions the final, important question should also be answered.

4.2 Methods

4.2.1 Preliminary testing

Isolates VdII, 90-02, 84020, STR3 and 115 were each inoculated at 1 x 10^6 spores per ml into 125 ml of PLY medium. Shake cultures were incubated at 20-25°C at 250 rpm for 1 week. Cultures were passed through two layers of muslin to separate mycelium from spores. Spores were washed with reverse osmosis water and the concentration adjusted to 1 x 10^6 per ml in a total volume of 50 ml. Equal concentrations of spores from isolates VdII, 90-02, and 84020 were mixed and adjusted to a final concentration of 1 x 10^6 spores per ml in 50 ml.

Three week old *Brassica napus* ssp. *oleifera* var. Mikado plants were removed from their pots (7cm x7cm x 8cm) and the soil carefully washed from the roots by submerging in water. Roots were blotted with tissue paper and approximately 2.5cm of root was cut away, and the roots remaining on the plants were immediately submerged into the spore suspension for 5minutes. STR3, 115, and the VdII/90-02/84020 crucifer isolate mix was replicated in three plants, with suitable controls of plants with cut roots that had been exposed solely to water and plants that had not been uprooted from their pots. Inoculated plants and controls were incubated in propagators on capillary matting with the vents closed. After 3 days vents were opened, and after 10 days propagator lids were removed. Plants were scored for incidence of wilt.

4.2.2 Cut-root test

For each isolate, 125ml PLY medium was inoculated with two cryo-beads from a frozen stock and incubated at 20-25°C at 250 rpm for 1 week. Cultures were passed through two

layers of muslin to separate mycelium from spores. Spores were washed in reverse osmosis water and the concentration adjusted to 1 x 10^6 per ml in a total volume of 150ml. Pathogenicity was confirmed for each isolate by inoculation into and re-isolation from the susceptible tomato (*Lycopersicon esculentum*) cultivar Santa.

Two week old *Brassical napus* ssp. *oleifera* var. Liaison plants were carefully removed from their pots (7cmx7cmx8cm) and excess soil removed from the roots by careful washing under water. Roots were blotted with tissue paper and approximately 2.5cm of root was cut away and the remaining roots submerged in the spore suspension for 5 minutes. Inoculated plants were repotted into pots (7cmx7cmx8cm)with Levingtons M2 compost, and placed into propagators on capillary matting with the vents closed. Vents were gradually opened over 4 days. Plants were then place in gravel trays on capillary matting in a split block design (Figure 4.4) (Table 4.1) (Dr J Jones- Warwick HRI). Disease scores were recorded at 10, 15, 22, and 28 d.p.i(days post inoculation) with the final height of the plant recorded at 37 d.p.i Final analysis used plant height at 37d.p.i and disease scores from 28d.p.i.

4.2.3 Infested soil

The preparation of the inoculum was adapted from the procedure of Atibalentja and Eastburn (1997). Whole oat seed (50 g per 200 ml of reverse osmosis water) was soaked overnight in glass honey jars and sterilised at 121°C at 15 p.s.i for 1hour. After excess liquor was poured off, the oat seed was incubated at room temperature overnight and then sterilised again at 121°C at 15 p.s.i for 1hour.

The contents of each flask were inoculated with 1 x 10^6 spores prepared by washing the surface of a 4 week-old culture on PLYA. Flasks were incubated in the dark at 20-25°C for 3 weeks. Some individual oat seeds were aseptically removed and placed on to water agar (10 g agar per litre RO water pH 5.8-6.0) and incubated in the dark for 1 week to confirm the fungus was viable on oat seed. Colonised oats were mixed with soil (Levingtons M2 compost) in at 1:3 ratio (by volume) and placed into pots (7cmx7cmx8cm). Individual seeds (one per pot) of *B. napus* ssp. *oleifera* var. Liaison or 1 cm segments of horseradish root (one per pot)were placed into the infested soil; controls were planted into soil alone or soil supplemented with uninoculated oat seed. Pots were then placed on capillary matting in gravel trays in a random plot block design (Dr J Jones, Warwick HRI) with each crop replicated twice in each plot and each isolate once with each crop in plot. There were 12 replicates per isolate per crop, two control treatments, of soil amended with uninoculated oat seed and soil on its own, each replicated 12 times within each crop (Figure 4.5) (Table 4.1). Disease scores were recorded when the oil seed rape plants grown in soil amended with uninoculated oat seed reached an mean average of 6cm, 12cm , 45cm and 80cm in height.

4.2.4 Disease score index

In both cut root and infested soil experiments, oilseed rape plants were scored as follows. 1=healthy, 2=one or two cotyledons chlorotic, 3=cotyledons fully chlorotic with some necrosis, 4=complete necrosis in cotyledons, 5=Partial chlorosis in lower adult leaves, 6=Full chlorosis in lower adult leaves and some necrosis, 7= Complete necrosis in some adult leaves, 8=plant death (Figures 4.6-4.8).

Horseradish plants were scored by foliar symptoms as follows. 1=healthy, 2=slight chlorosis limited to one leaf, 3=two to less than 25% of the leaves with chlorosis and necrosis, 4=25% to less than 50% of the leaves with chlorosis and necrosis, 5=50% to less than 75% of the leaves with chlorosis and necrosis, 6=75% or more leaves with chlorosis and necrosis, 7=Almost 100% leaves necrotic, 8=plant death. (adapted from Atibalentja and Eastburn, 1998). Petiole length was also measured for leaves that were not necrotic, and where necessary the average recorded (Figures 4.9 and 4.10).

4.2.5 Re-isolation of fungus from plant tissue

Where possible 3cm sections of petiole were taken and washed in 70% ethanol for 5minutes with occasional shaking. The sections of petiole were air-dried, approximately 0.5cm removed from each cut end and the main piece placed on to PLYA, incubated in the dark at 20-25°C.

1. Do amphihaploid isolates of *Verticillium* from crucifers differ from haploid *Verticillium* isolates in their pathogenicity towards a single cruciferous crop?			
Crop	Isolates	Replicates per isolate	Method of inoculation
Brassica napus* ssp. *oleifera	**20 (inc. 2 control groups)**	**6**	**Cut-root**

2. Are crucifer isolates host-specific?			
Crop	Isolates	Replicates per isolate	Method of inoculation
Brassica napus* ssp. *oleifera	**14 (inc. 2 control groups)**	**12**	**Infested soil**
Armoracia rusticana	**14 (inc. 2 control groups)**	**12**	**Infested soil**

Table4.1. Summary of experimental procedures used in main plant inoculation experiments

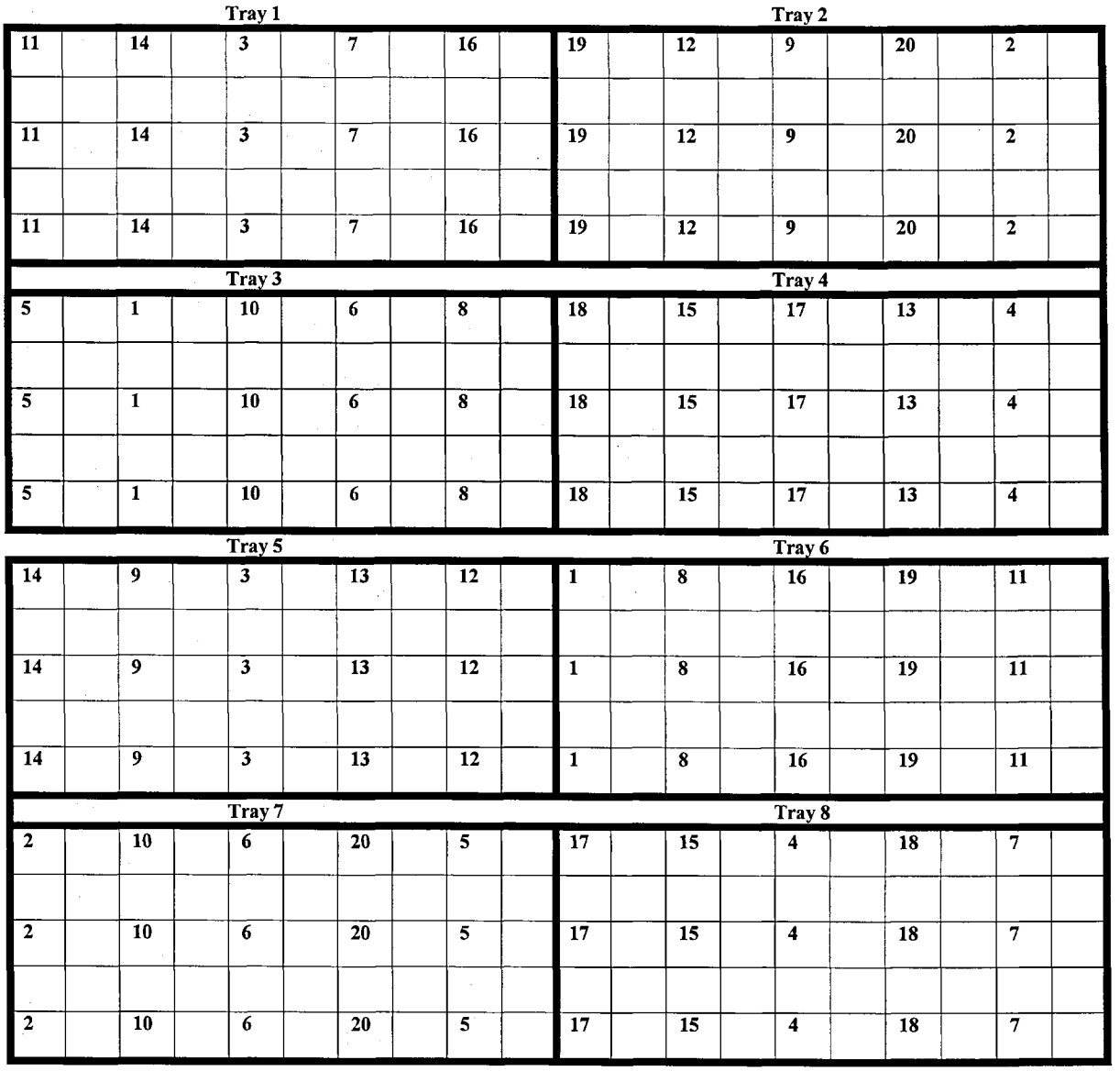

Figure 4.4. Layout of experiment for cut-root test on oilseed rape. Numbers represent each treatment (isolate or control). Squares without numbers were spaces. Six replicates per isolate were tested.

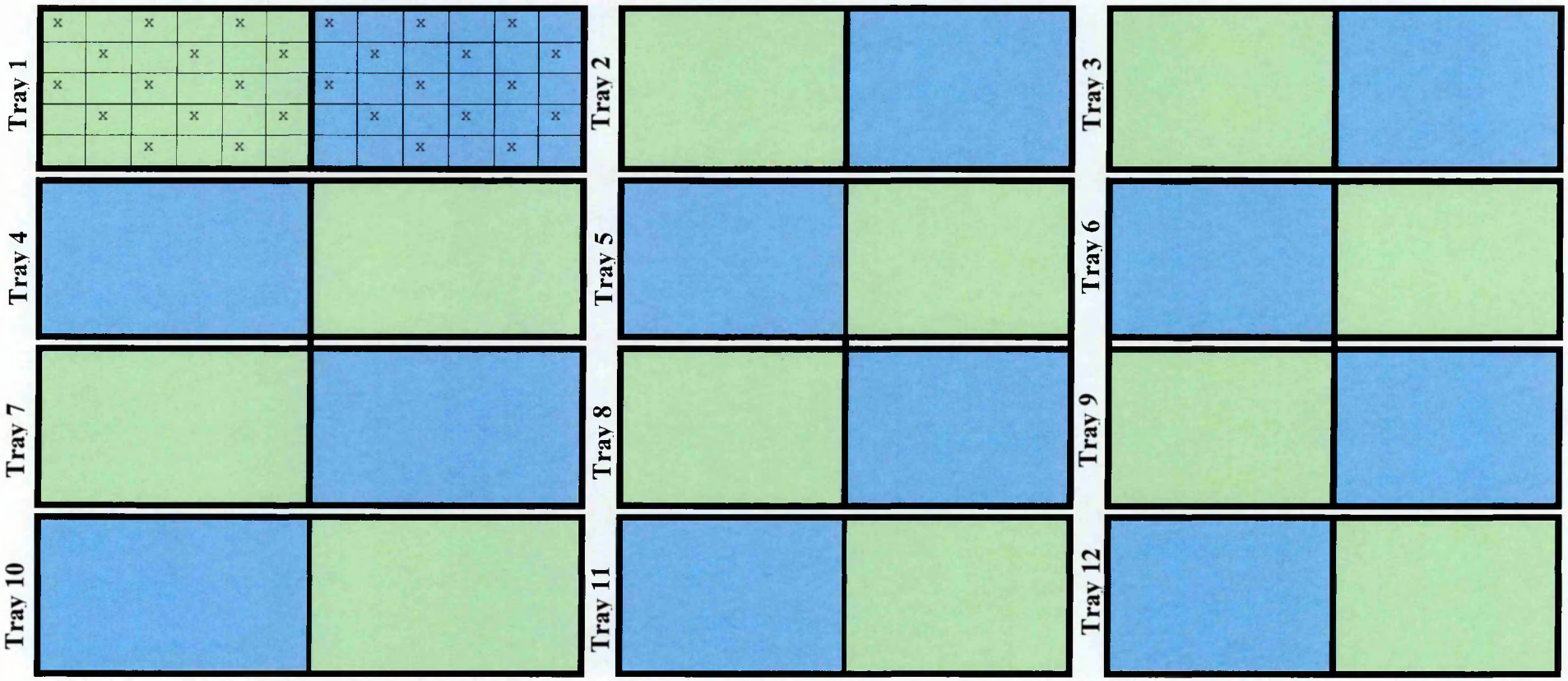

Figure 4.5. Layout of experiment for infested soil tests on oilseed rape and horseradish. ☐ Shaded squares are oilseed rape plots ☐ shaded squares are horseradish plots. Tray 1 gives and example of how the plants were laid out, each cross represent a plant infected with one isolate. Squares unchecked represent spaces. Each isolate was represented once in each plot, and each replicated 12 times per isolate per crop. Positions of individual isolates were randomised within plots.

Isolate	Host	AFLP group[a]	CR1	CR2	CR3	CR4	IF
12080	Soil	*V. dahliae*[1]	✓	✓	✓	✓	
12087	*Fragaria anannassa* (strawberry)	*V. dahliae*[1]					✓
Md71	*Matricaria chamomilla* (chamomile)	*V. dahliae*[1]		✓	✓		
P14	*Lycopersicon esculentum* (tomato)	*V. dahliae*[1]	✓	✓	✓	✓	✓
Vd128	*Brassica oleracea* ssp. *botrytis* (broccoli)	*V. dahliae*[2]	✓	✓	✓	✓	✓
Md80	*B. napus* ssp. *oleifera* (oilseed rape)	*V. dahliae*[2]	✓	✓	✓	✓	✓
001	*Armoracia rusticana* (horseradish)	β	✓	✓	✓	✓	✓
004	*Armoracia rusticana*	β	✓	✓	✓	✓	
Md73	*B. napus* ssp. *oleifera*	β	✓	✓	✓	✓	✓
9802	*Armoracia rusticana*	β	✓	✓	✓	✓	✓
Vd292	*B. oleracea* ssp. *botrytis* (cauliflower)	α	✓			✓	
VdII	*B. napus* ssp. *oleifera*	α	✓	✓	✓	✓	✓
84020	*B. campestris* ssp. *rapifera* (turnip)	α	✓			✓	✓
90-10	*B. oleracea* ssp. *botrytis*	α	✓	✓	✓	✓	✓
KRS1	*Medicago sativa* (lucerne)	*V. albo-atrum* (L)	✓	✓	✓	✓	
STR1	*Medicago sativa*	*V. albo-atrum* (L)	✓	✓	✓	✓	✓
VA1	*Lycopersicon esculentum*	*V. albo-atrum* (NL)	✓	✓	✓	✓	
1974	*Humulus lupulus* (hop)	*V. albo-atrum* (NL)		✓	✓		✓
11041	*Humulus lupulus*	*V. albo-atrum* (NL)	✓	✓	✓	✓	
151	Soil	*V. albo-atrum* (GpII)	✓	✓	✓	✓	
1988	*Lycopersicon esculentum*	*V. tricorpus*	✓	✓	✓	✓	

Table 4.2. Isolates used in main pathogenicity testing. [a]AFLP groups defined by Collins *et al.* (2003), *V. dahliae*[1] haploid isolates not from Brassicaceae, *V. dahliae* [2] haploid isolates from Brassicaceae (Barbara and Clewes, 2003) referred to as secondary haploid in Collins *et al.* (2003). CR1-4 indicates each cut-root test and the isolates used, IF indicated the infested soil experiment and the isolates used.

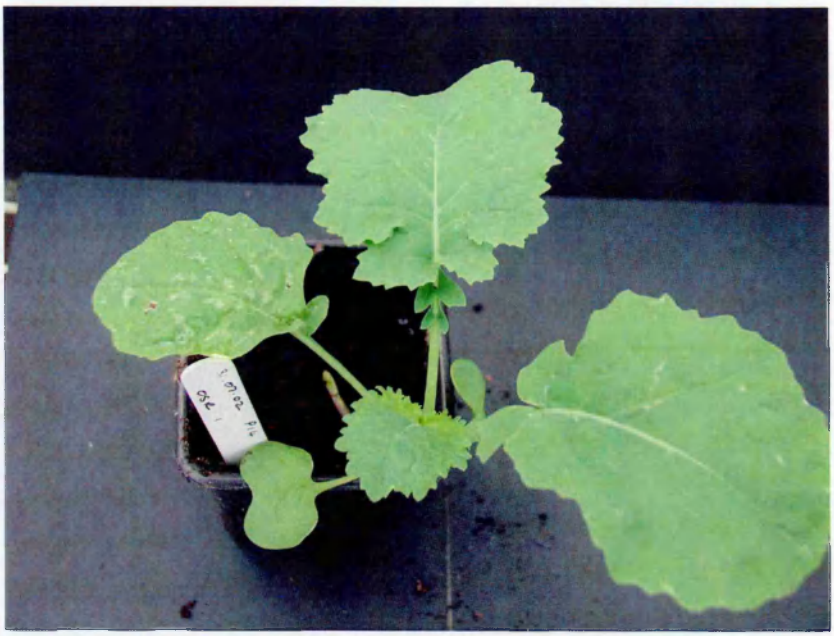

Figure 4.6. Asymptomatic oilseed rape plant infected with *Verticillium*

Figure 4.7. Infected oilseed rape plant with necrotic cotyledons and partial chlorosis in lower adult leaves

Figure 4.8. Infected Oilseed rape plant with chlorotic lower adult leaves

Figure 4.9. Horseradish plant infected with *Verticillium* with chlorosis in 25-50% of leaves

Figure 4.10. Horseradish plant with necrosis in more than 75% of leaves.

4.3 Results

4.3.1 Preliminary testing

Severe wilt was evident in plants inoculated with the mixture of spores from three crucifer isolates (Figure 4.11). Plants inoculated with spores from *V. albo-atrum* STR3 showed symptoms of wilt (Figure 4.12). Plants inoculated with *V. dahliae* 115 did not show symptoms of wilt (Figure 4.13) as did plants that were not inoculated with spores but just dipped into sterile distilled water did not show any symptoms (Figure 4.14). Fungus was re-isolated from inoculated asymptomatic and symptomatic plants and the species identity of the isolate was confirmed for plants inoculated with STR3 and 115 by PCR using primers ITS4/361 and ITS4/363 (data not shown).

4.3.2 Cut-root inoculation

Each separate test (Figure 4.15-4.18) separated the amphihaploid isolates from the *V. dahliae* non-cruciferous isolates, although maybe not to as great a degree as expected Figure 4.15 shows the best separation of isolates according to score and height. Here, there are three broad groups, representing isolates from AFLP group α, AFLP group β and haploid *V. dahliae* isolates from non-cruciferous hosts, this is to some lesser extent replicated in the following three Figures (4.16, 4.17, and 4.18). It is important to note that *V. dahliae* isolates from cruciferous hosts (Vd128 and Md80) were not consistent in their pathogenicity towards oilseed rape in the tests, in two of the four figures being different from each other.

Results for the control plants were similarly grouped in each Figure representing each test. In each instance it is apparent the controls that had roots cut away and then dipped into water were shorter in height than those that had been left in their pots, un-cut. It must also be pointed out that the control plants were also scored, and in three of the four tests that mean scores above that of 'healthy' (1) were found, why this should be is not known but it may be postulated that some symptoms correspond with the natural process of aging, and as such could be confused as such with typical symptoms of *Verticillium* infection.

In Figure 4.15 isolates from AFLP group α fall relatively closely together, in ranging disease score from 4-6 and from, and 30-56cm mean height, group β isolates were more widely found in terms of height (41-79cm) but similar to the AFLP group α isolates in terms of disease score. This is repeated in the following Figures but to a lesser extent. Overall it can be seen that the α group isolates give more stunting, but only a little greater disease than the β groups. Both AFLP groups could be distinguished from the non-cruciferous haploid *V. dahliae* isolates in all four tests. However, this 'distinction' of two populations is by not clear cur and should be seen as tentative as it is based only on a few isolates.

Isolates of *V. albo-atrum* (both Lucerne and Non-Lucerne pathotypes) gave no obvious groupings with each other or with isolates of the two other species, *V. albo-atrum* (GpII) (isolate 151), and the isolate of *V. tricorpus* (1988). Fungus was re-isolated from all isolates tested and their being *Verticillium* species confirmed by the production of resting structures (data not shown).

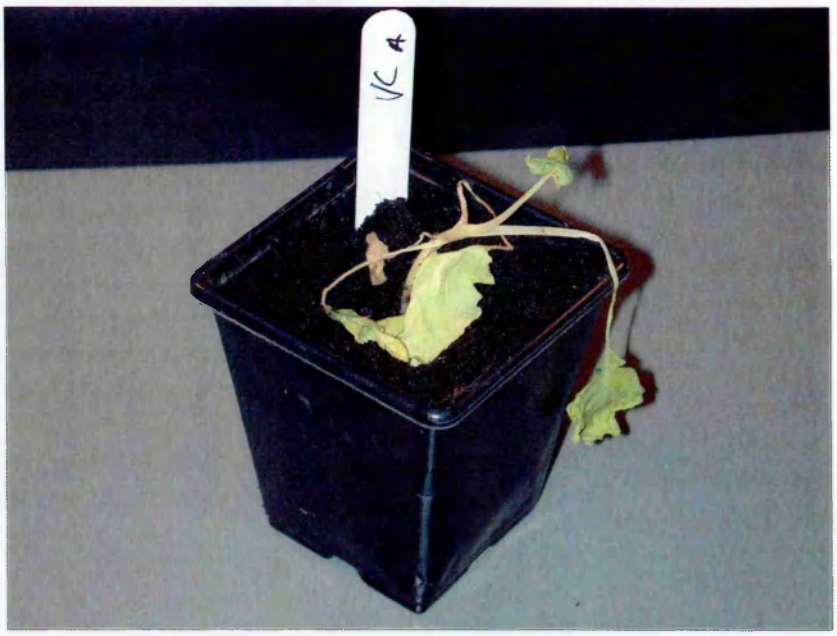

Figure 4.11. *B. napus* ssp. *oleifera* var. Mikado inoculated with spores from three *Verticillium* crucifer isolates from the preliminary experiment.

Figure 4.12. *B. napus* ssp. *oleifera* var. Mikado inoculated with *V. albo-atrum* isolate STR3 from the preliminary experiment.

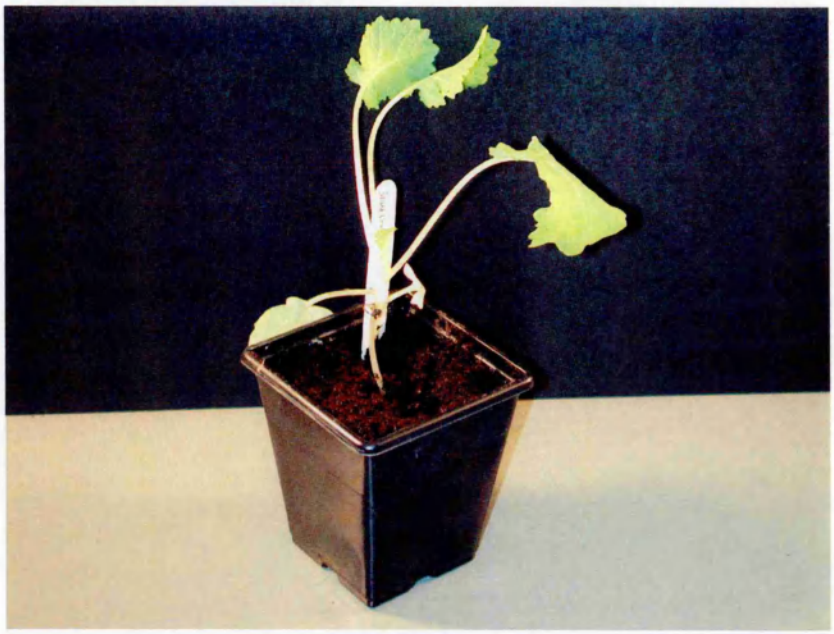

Figure 4.13. *B. napus* ssp. *oleifera* var. Mikado inoculated with *V. dahliae* isolate 115 from the preliminary experiment.

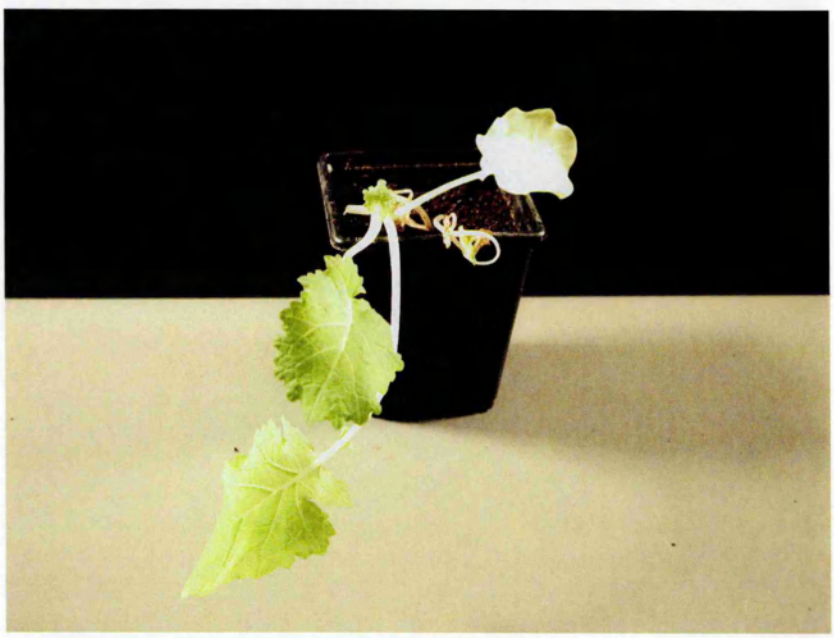

Figure 4.14. *B. napus* ssp. *oleifera* var. Mikado control from the preliminary experiment.

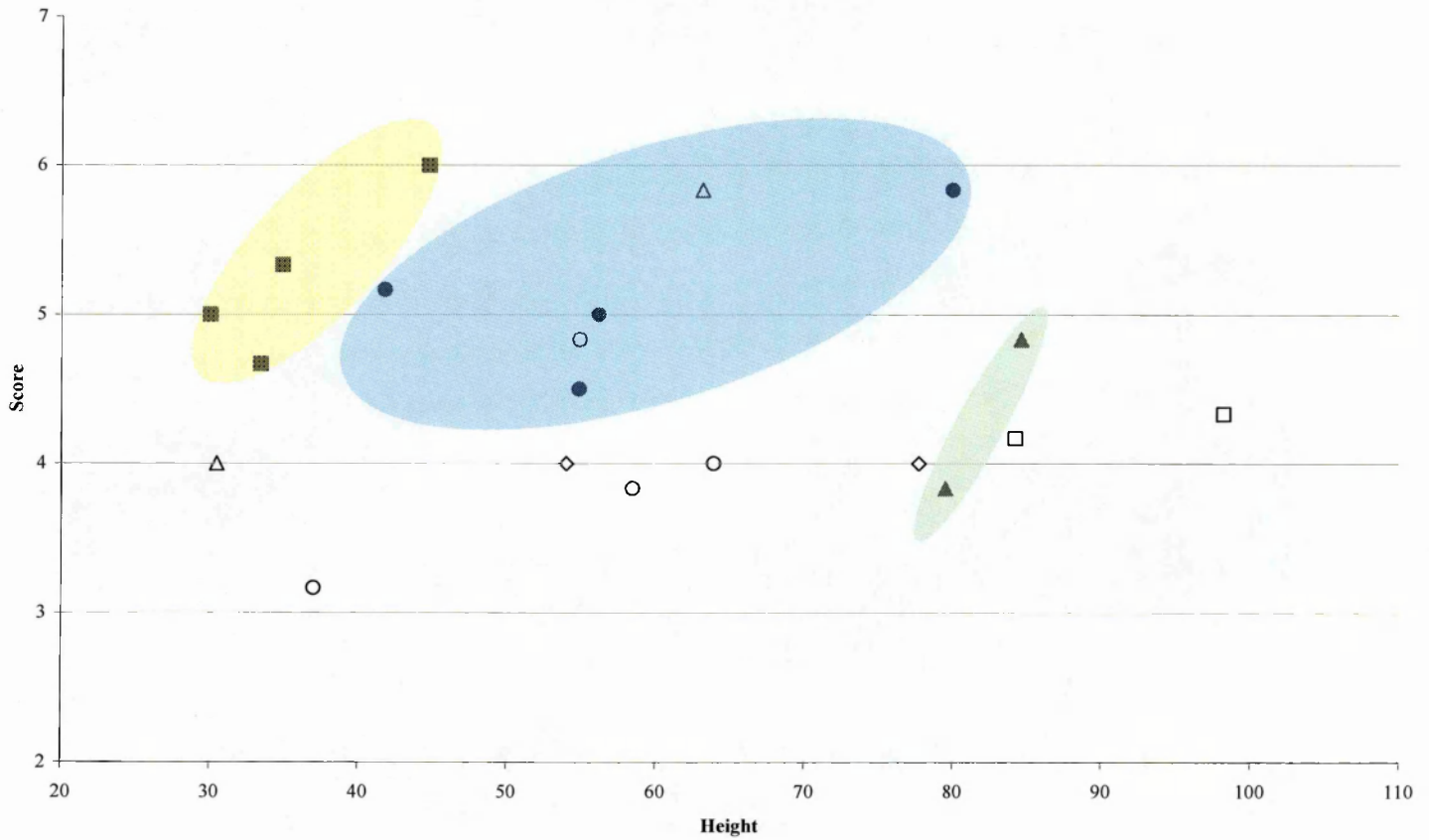

Figure 4.15. Results taken from first cut-root experiment. Oilseed rape height (cm) vs. Oilseed rape scores. ■ isolates from AFLP group α, ● isolates from AFLP group β, ▲ *V. dahliae* non-cruciferous isolates, □ control, ○ *V. albo-atrum* isolates, △ *V. dahliae* isolates from crucifers, ◇ isolates of *V. tricorpus* and *V. albo-atrum* (GpII). Shaded areas cover the three main groups of interest *viz.* AFLP groups α and β and the non-cruciferous haploid isolates.

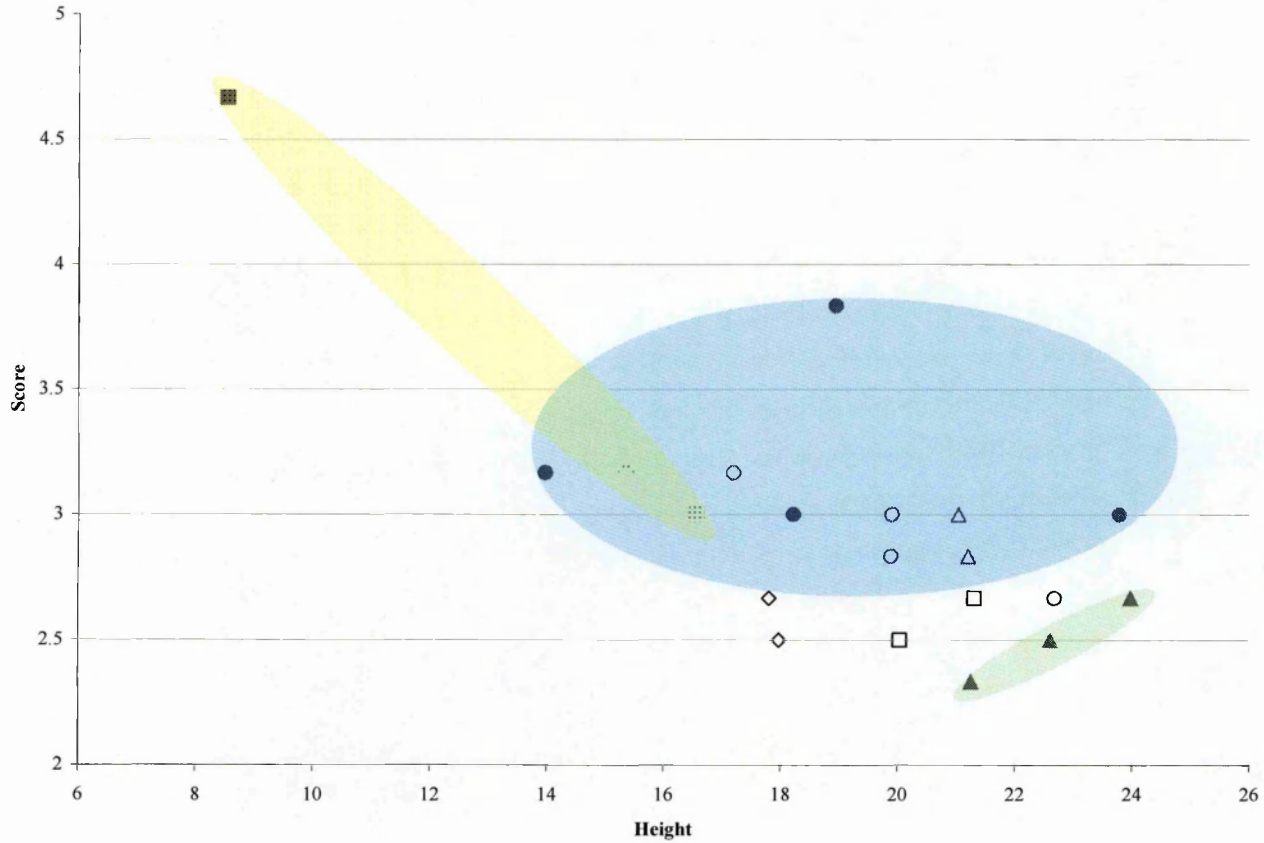

Figure 4.16. Results taken from second cut-root experiment. Oilseed rape height (cm) vs. Oilseed rape scores. ■ isolates from AFLP group α, ● isolates from AFLP group β, ▲ *V. dahliae* non-cruciferous isolates, □ control, ○ *V. albo-atrum* isolates, △ *V. dahliae* isolates from crucifers, ◇ isolates of *V. tricorpus* and *V. albo-atrum* (GpII).). Shaded areas cover the three main groups of interest *viz.* AFLP groups α and β and the non-cruciferous haploid isolates.

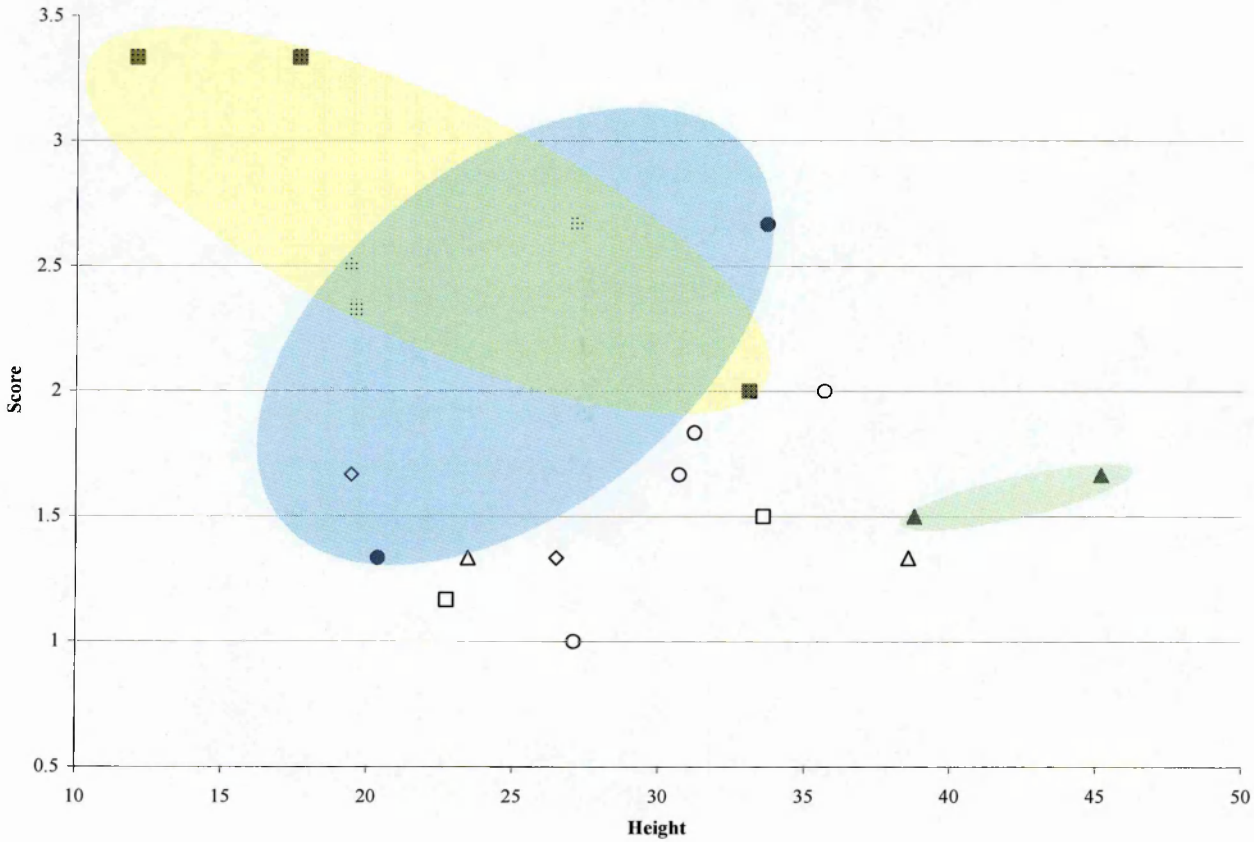

Figure 4.17. Results taken from third cut-root experiment. Oilseed rape height (cm) vs. Oilseed rape scores. ■ isolates from AFLP group α, ● isolates from AFLP group β, ▲ *V. dahliae* non-cruciferous isolates, □ control, ○ *V. albo-atrum* isolates, △ *V. dahliae* isolates from crucifers, ◇ isolates of *V. tricorpus* and *V. albo-atrum* (GpII). Shaded areas cover the three main groups of interest *viz.* AFLP groups α and β and the and the non-cruciferous haploid isolates.

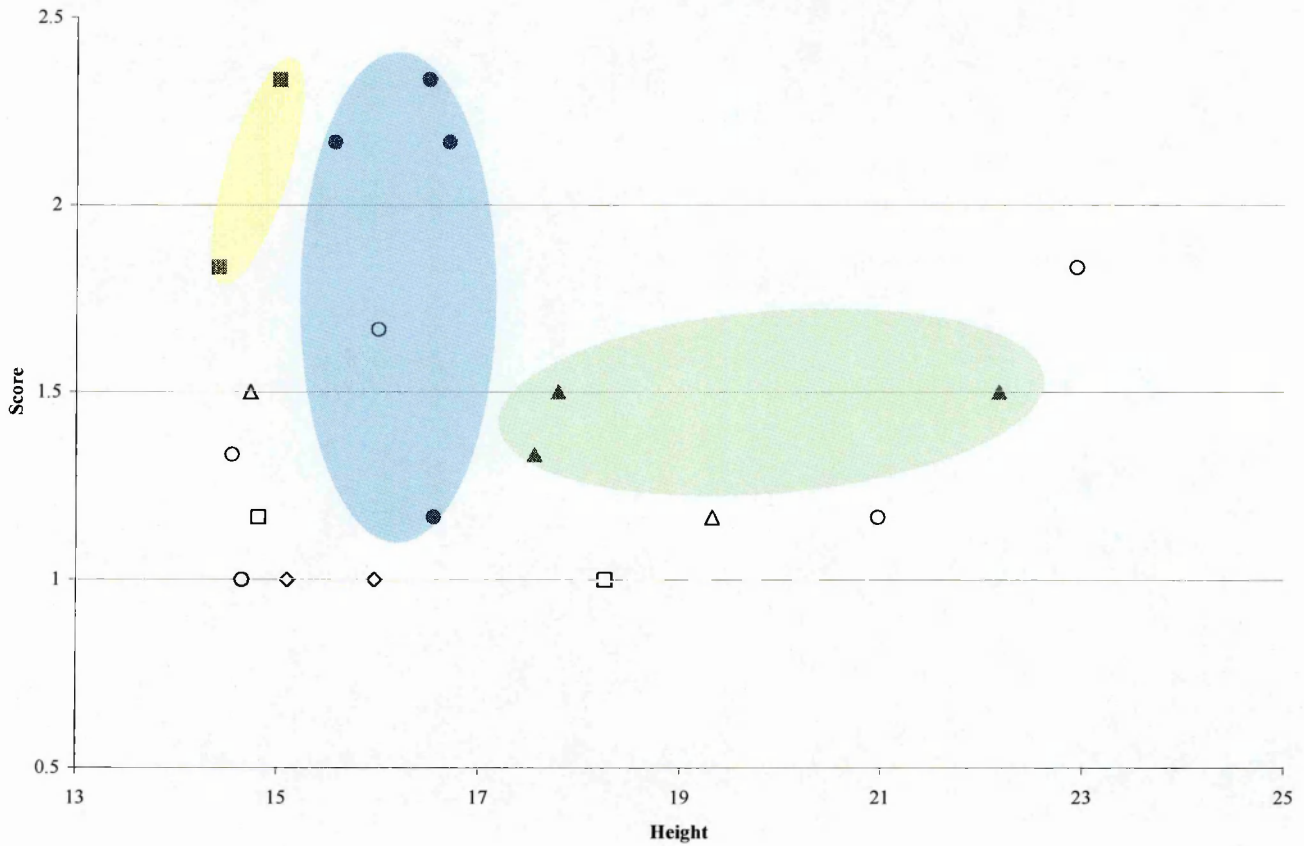

Figure 4.18. Results taken from fourth cut-root experiment. Oilseed rape height (cm) vs. Oilseed rape scores. ■ isolates from AFLP group α, ● isolates from AFLP group β, ▲ *V. dahliae* non-cruciferous isolates, □ control ○ *V. albo-atrum* isolates, △ *V. dahliae* isolates from crucifers, ◇ isolates of *V. tricorpus* and *V. albo-atrum* (GpII). Shaded areas cover the three main groups of interest *viz.* AFLP groups α and β and the and the non-cruciferous haploid isolates.

4.3.3 Infested soil

Verticillium was found to grow on and from oat seed as shown in Figure 4.19a and b. As experiments were conducted on seeds and cuttings, results were shown for all plants, and thus taking into account plants that did not grow, and plants that grew alone *i.e.* plants that produced shoots and leaves alone and thus disregarding plants that did not grow. Failure to grow may of course have been due to the presence of *Verticillium* in the soil, it may also be due to the presence of other soil-borne pathogens such as *Pythium*, which causes root rot.

For oilseed rape inoculation tests no one treatment achieved 100% germination, including controls (only 66.7 and 91.7% for with and without oat seed respectively) (Table 4.3). The lowest germination rate for oilseed rape was 66.7% seen in the with oat seed control and also pots infested with 84020, Md73 and VdII. For horseradish, the with and without oat seed controls achieved 75 and 100% growth respectively, and cuttings infected with isolates 12087, 9010 and 84020 also gave 100% growth. The lowest proportion of cuttings growing was in pots infested with isolate Md80 (16.7%).

Re-isolations were attempted from all plants in this study; all attempts but one were heavily contaminated with bacteria and failed to produce resting structures upon incubation. The one isolate that successfully produced microsclerotia was from an oilseed rape plant grown in soil infested with oat seed inoculated with isolate VdII.

For both sets of control plants results were variable. This data (Figures 4.20-4.31)showed that for oil seed rape plants the addition of oat seed to the soil decreased 'symptoms' but gave inhibition of height as compared to plants grown in soil without oat seed. Horseradish

was less consistent in they gave the same symptom score for all plants, but the mean symptom score for only the plants that grew was higher for plants grown in soil amended with oat seed than those grown in soil without. Similarly, the addition of oat seed to the soil gave shorter mean petiole length than those plants grown in soil without oat seed.

Scatter diagrams (Figure 4.20-4.31) drawn for all possible data combinations (Table 4.4), showed that these results were inconclusive, and gave little indication of host-specificity. Some degree of separation between AFLP groups α and β and the non-cruciferous *V. dahliae* isolates was observed (4.20-4.21) when oilseed rape height was plotted against disease score, but this was weak at best and by no means as defined as that observed from the cut-root tests. The best result that may be an indication for this was the comparison for all plants between horseradish scores and oilseed rape height (Figure 4.28). For all plants the correlation between oilseed rape height and horseradish score was -0.49, it could be argued that this is similar to the results from the cut root tests, it is however ambiguous in the distinction of two populations between amphihaploid and haploid isolates, and as such this test could not be used to reliably distinguish an artificial amphihaploid from a non-cruciferous *V. dahliae* isolate.

One way ANOVA (without blocking) showed that for all plants of horseradish for the variates disease score and length of petiole there were significant difference between the isolates and controls (F.pr.<0.001) (Table 4.5 and 4.7). This was not true for oilseed rape using both variates. The variate disease score (F.pr. 0.385) and height (F.pr.0.112) showed that there were no significant differences (Table 4.8 and 4.9).

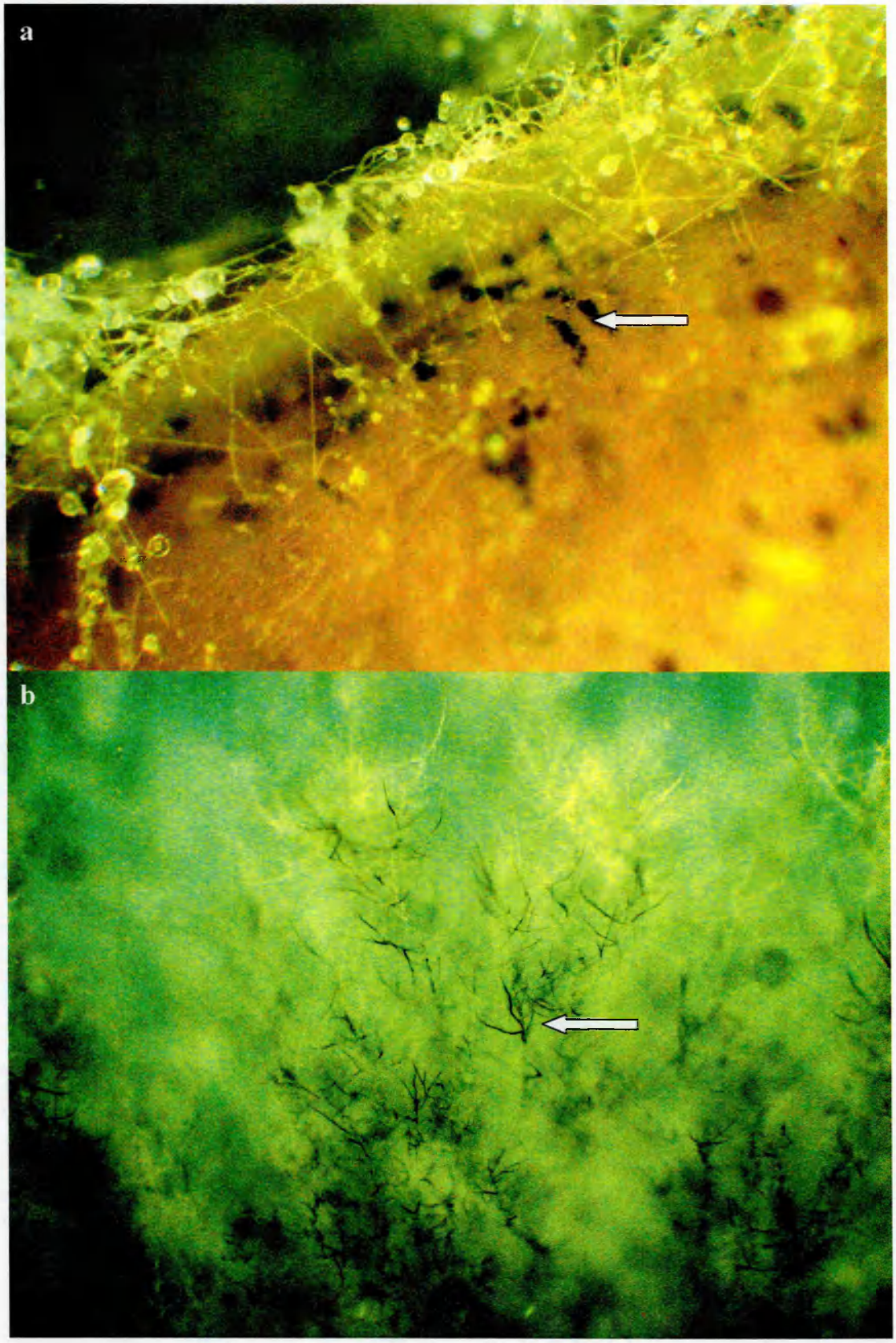

Figure 4.19a) *V. dahliae* growing on oat seed, arrow points to microsclerotia b) *V. albo-atrum* growing away from oat seed on water agar, arrow points to dark resting mycelium. Colour adjusted using Microsoft® Photo Editor® 2002.

	% germination/growth of cuttings													
Isolate/Control	**Plus**	**Without**	**12080**	**P14**	**1974**	**STR1**	**Vd128**	**Md80**	**VdII**	**9010**	**84020**	**Md73**	**001**	**9802**
Oilseed rape	66.7	91.7	83.4	75	91.7	83.4	83.33	75	66.7	75	66.7	66.7	75	91.7
Horseradish	75	100	100	50	91.7	25	58.4	16.7	66.7	100	100	66.7	75	66.7

Table 4.3. Percentage germination or growth of cuttings of each crop, with each isolate/controls.

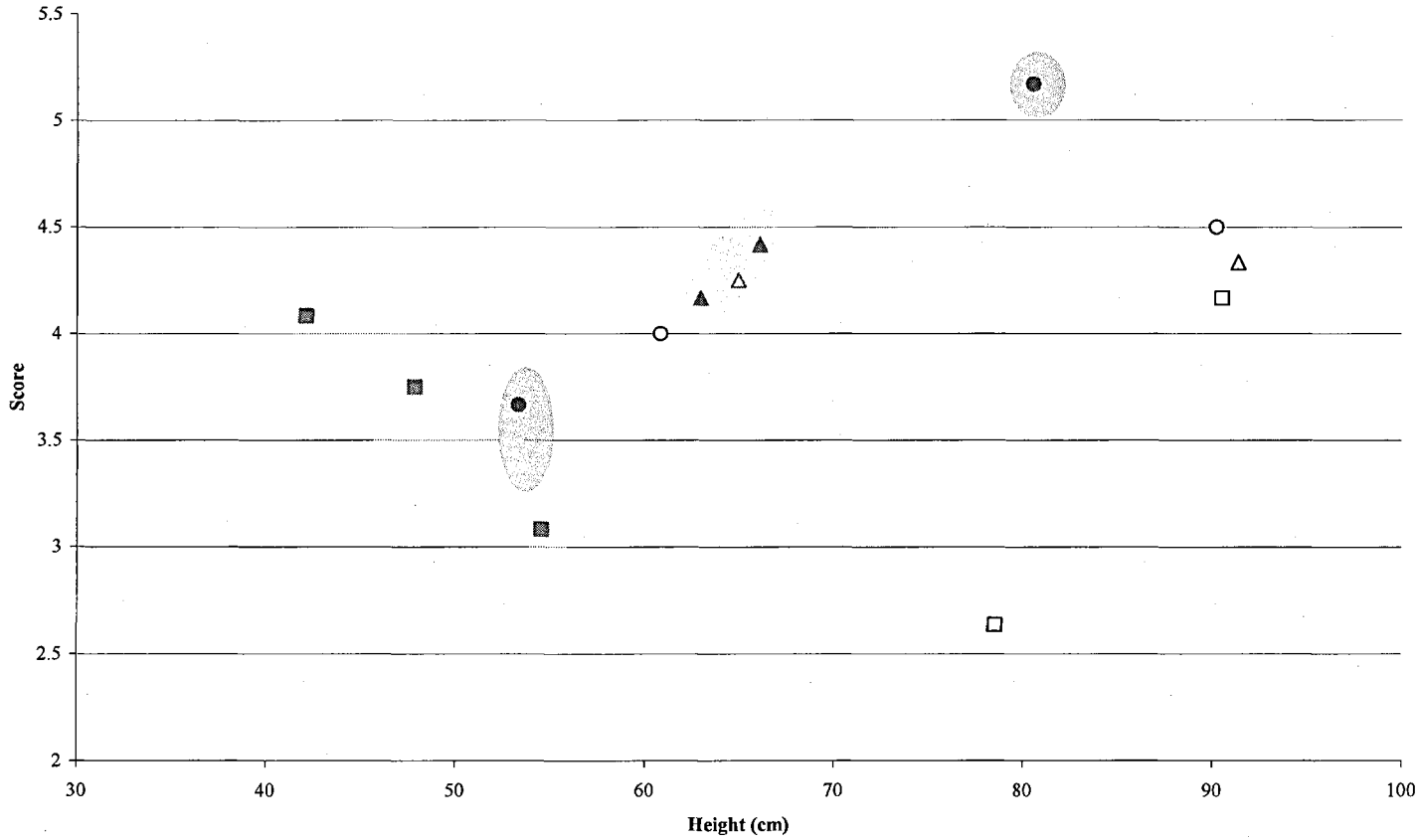

Figure 4.20. Results taken from infested soil experiment for all plants. Oilseed rape height (cm) vs. Oilseed rape scores. ■ isolates from AFLP group α, ● isolates from AFLP group β, ▲ *V. dahliae* non-cruciferous isolates, □ control, ○ *V. albo-atrum* isolates, △ *V. dahliae* isolates from crucifers. Shaded areas cover the three main groups of interest *viz.* AFLP groups α and β and the and the non-cruciferous haploid isolates.

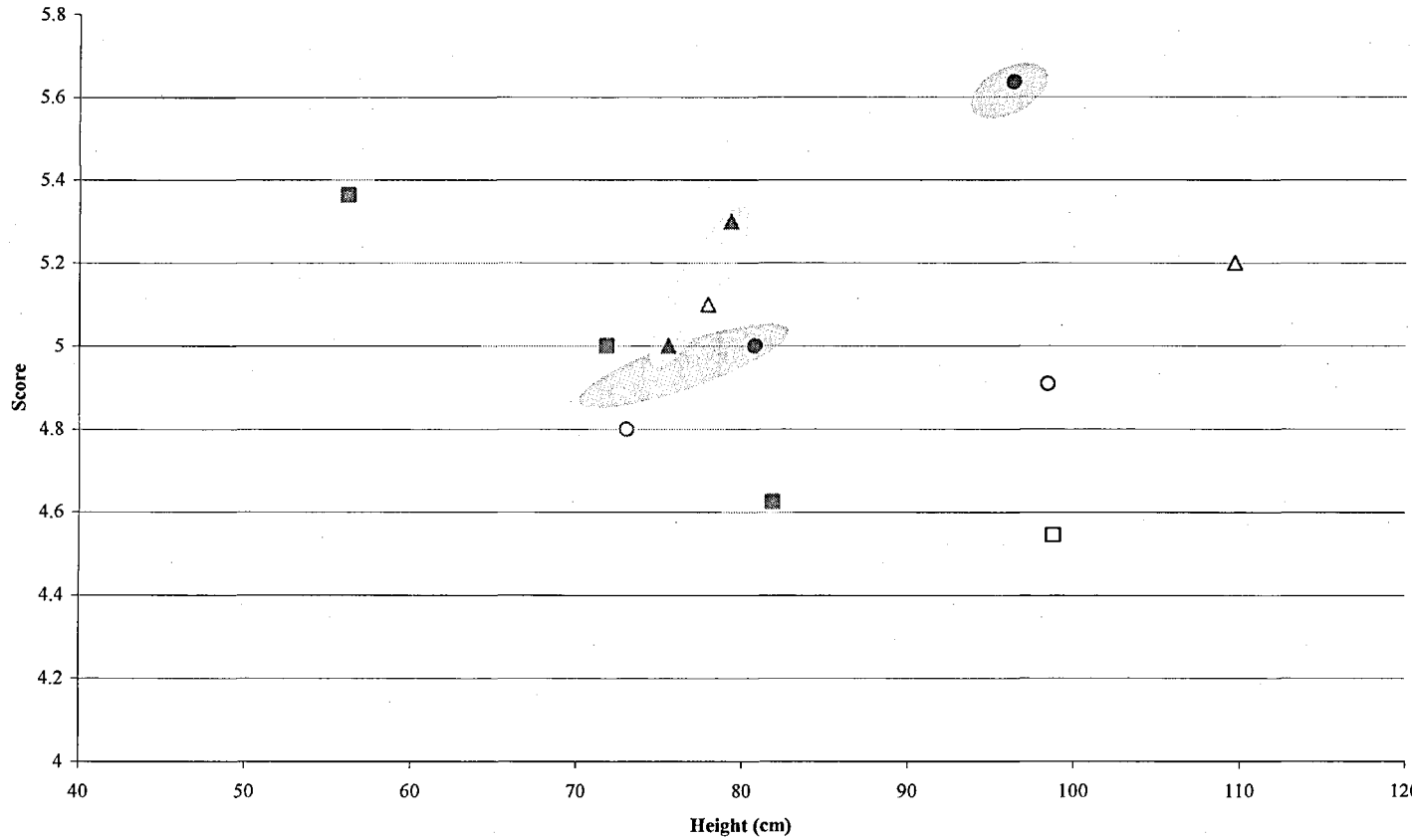

Figure 4.21. Results taken from infested soil experiment for only plants that grew. Oilseed rape height (cm) vs. Oilseed rape scores. ■ isolates from AFLP group α, ● isolates from AFLP group β, ▲ *V. dahliae* non-cruciferous isolates,□ control, ○ *V. albo-atrum* isolates, △ *V. dahliae* isolates from crucifers. Shaded areas cover the three main groups of interest *viz.* AFLP groups α and β and the and the non-cruciferous haploid isolates.

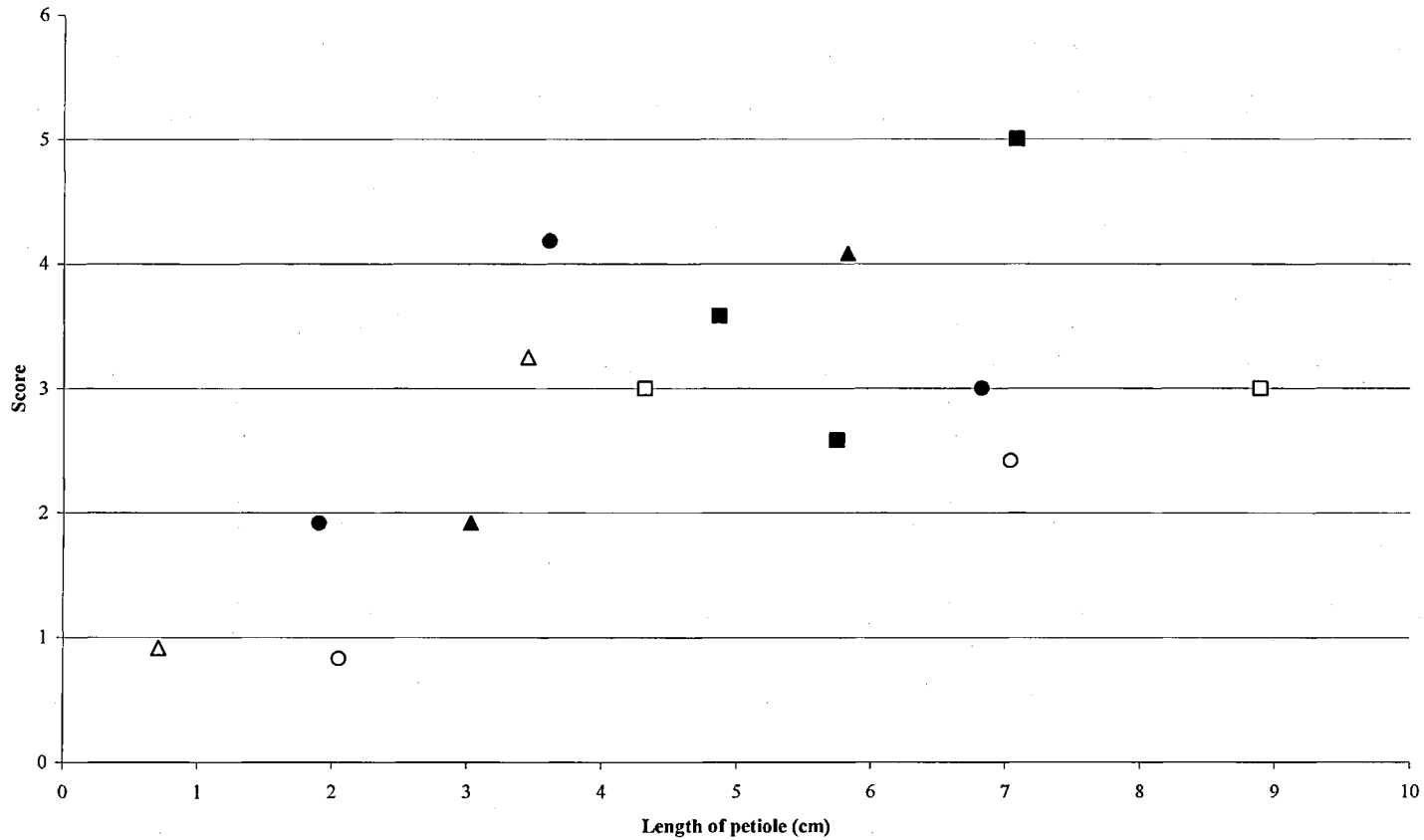

Figure 4.22. Results taken from infested soil experiment for all plants. Horseradish length of petiole (cm) vs. horseradish score. ■ isolates from AFLP group α, ● isolates from AFLP group β, ▲ *V. dahliae* non-cruciferous isolates, □ control, ○ *V. albo-atrum* isolates, △ *V. dahliae* isolates from crucifers. Shaded areas cover the three main groups of interest *viz.* AFLP groups α and β and the and the non-cruciferous haploid isolates.

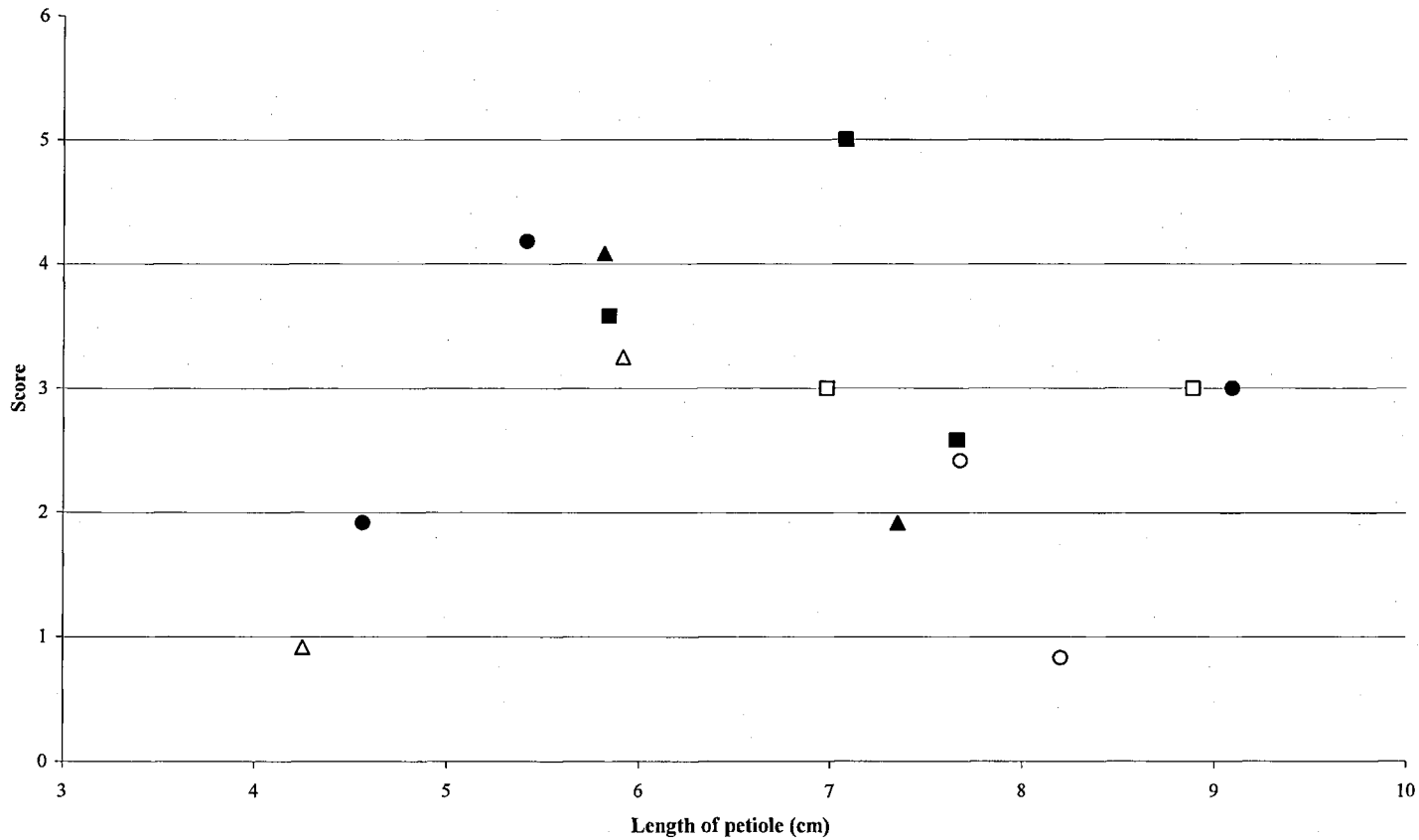

Figure 4.23. Results taken from infested soil experiment for only plants that grew. Horseradish length of petiole (cm) vs. horseradish score. ■ isolates from AFLP group α, ● isolates from AFLP group β, ▲ *V. dahliae* non-cruciferous isolates, □ control, ○ *V. albo-atrum* isolates, △ *V. dahliae* isolates from crucifers.

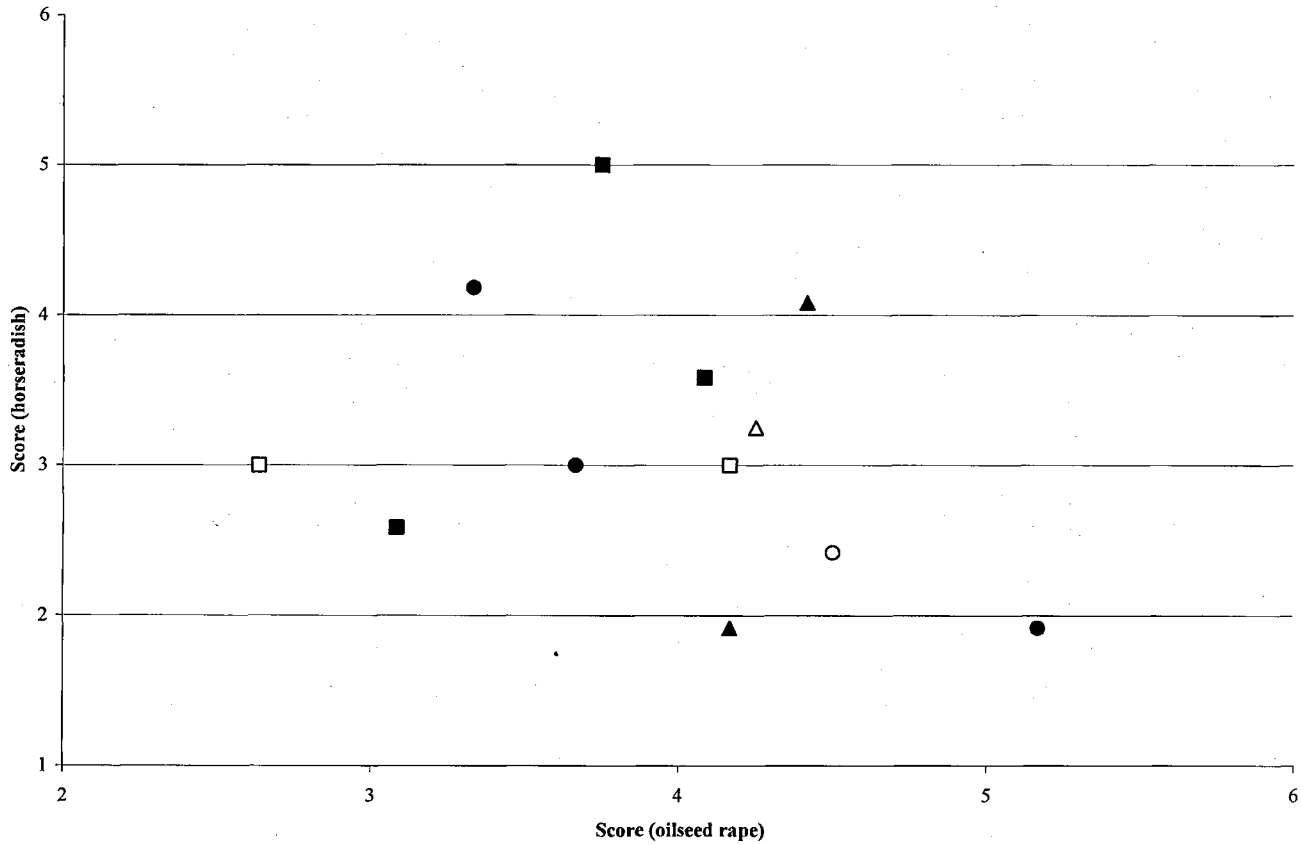

Figure 4.24. Results taken from infested soil experiment for all plants . Score (oilseed rape) vs. Score (horseradish). ■ isolates from AFLP group α, ● isolates from AFLP group β, ▲ *V. dahliae* non-cruciferous isolates, □ control, ○ *V. albo-atrum* isolates, △ *V. dahliae* isolates from crucifers.

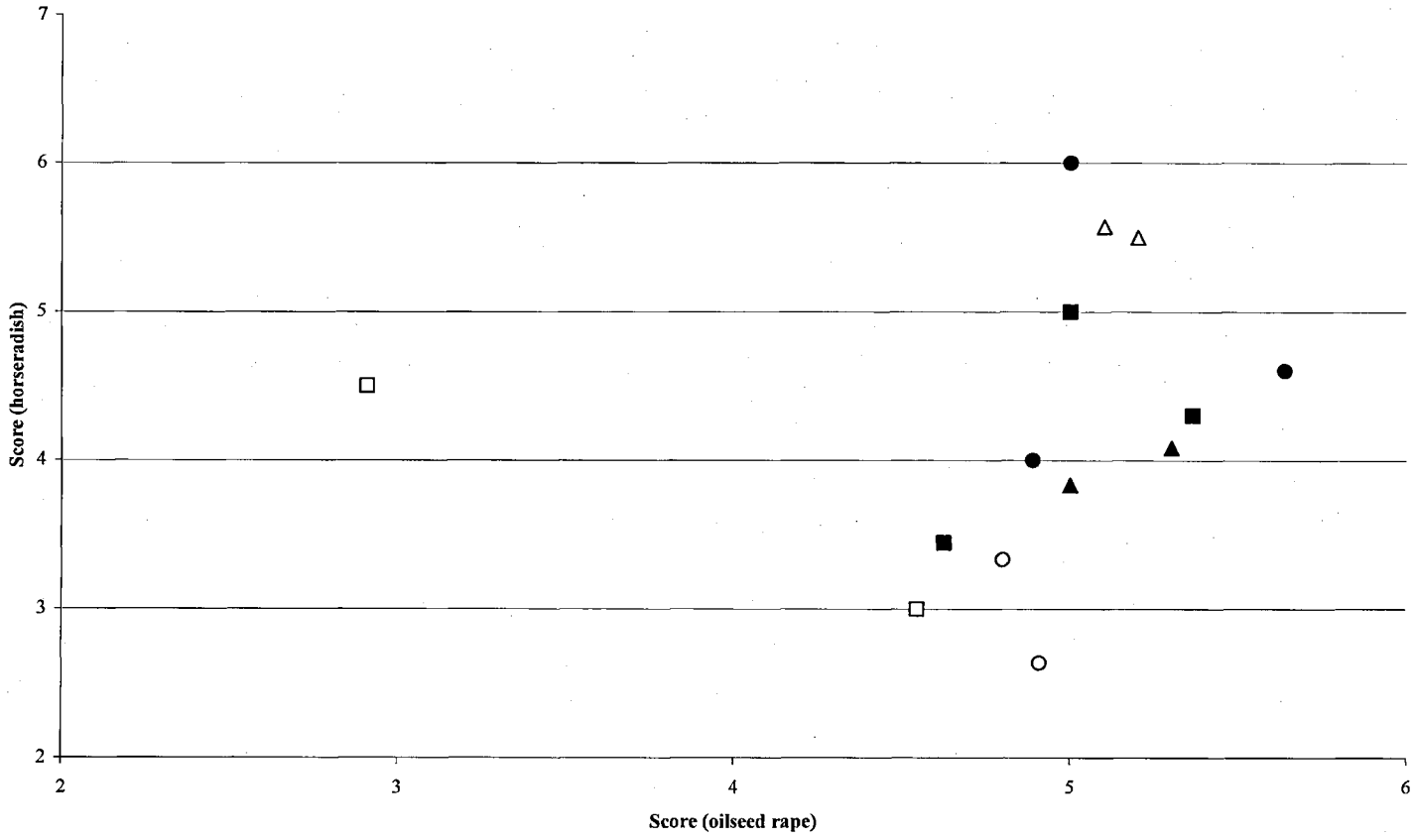

Figure 4.25. Results taken from infested soil experiment for only plants that grew. Score (oilseed rape) vs. Score (horseradish). ■ isolates from AFLP group α, ● isolates from AFLP group β, ▲ *V. dahliae* non-cruciferous isolates, □ control, ○ *V. albo-atrum* isolates, △ *V.*

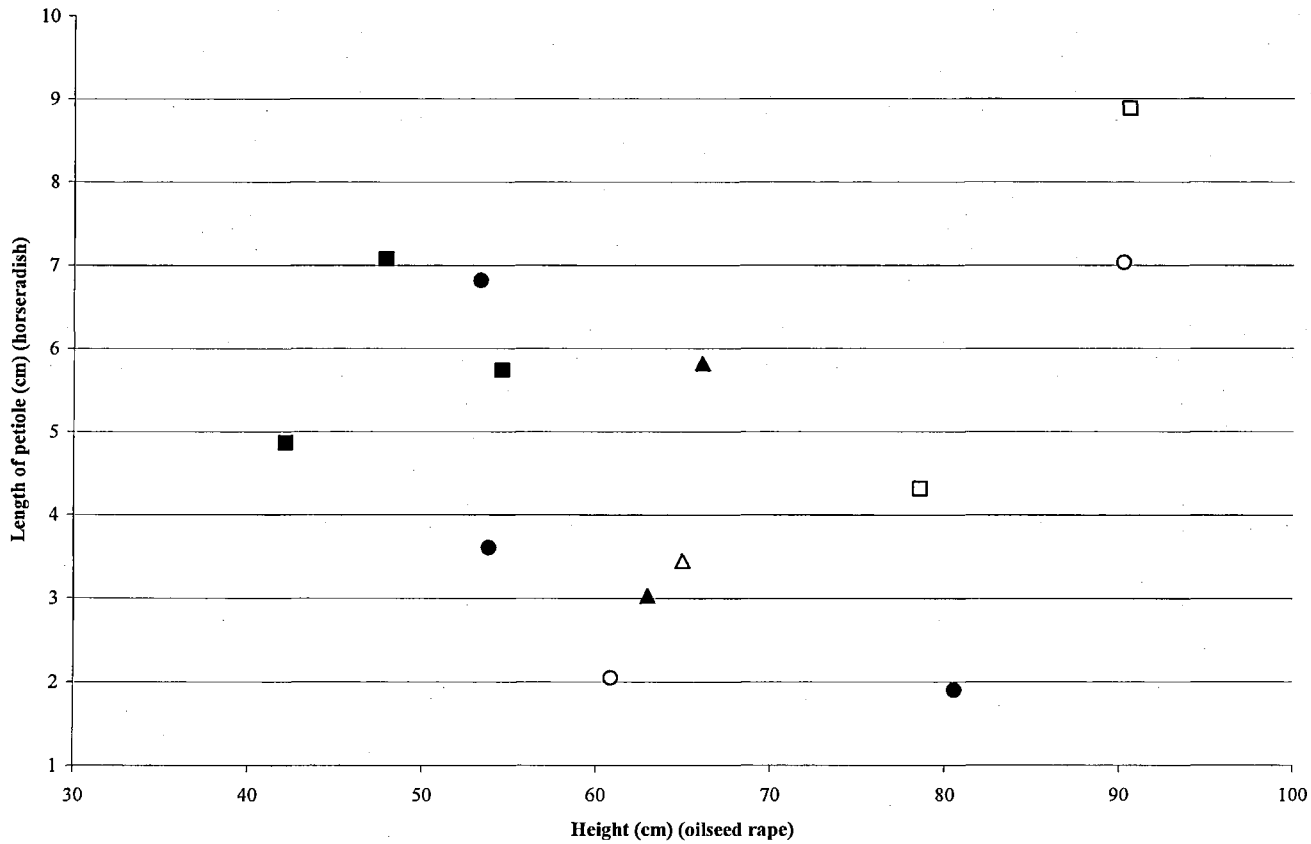

Figure 4.26. Results taken from infested soil experiment for all plants. Height (cm) (oilseed rape) vs. Length of petiole (cm) (horseradish). ■ isolates from AFLP group α, ● isolates from AFLP group β, ▲ *V. dahliae* non-cruciferous isolates, □ control, ○ *V. albo-atrum* isolates, △ *V. dahliae* isolates from crucifers.

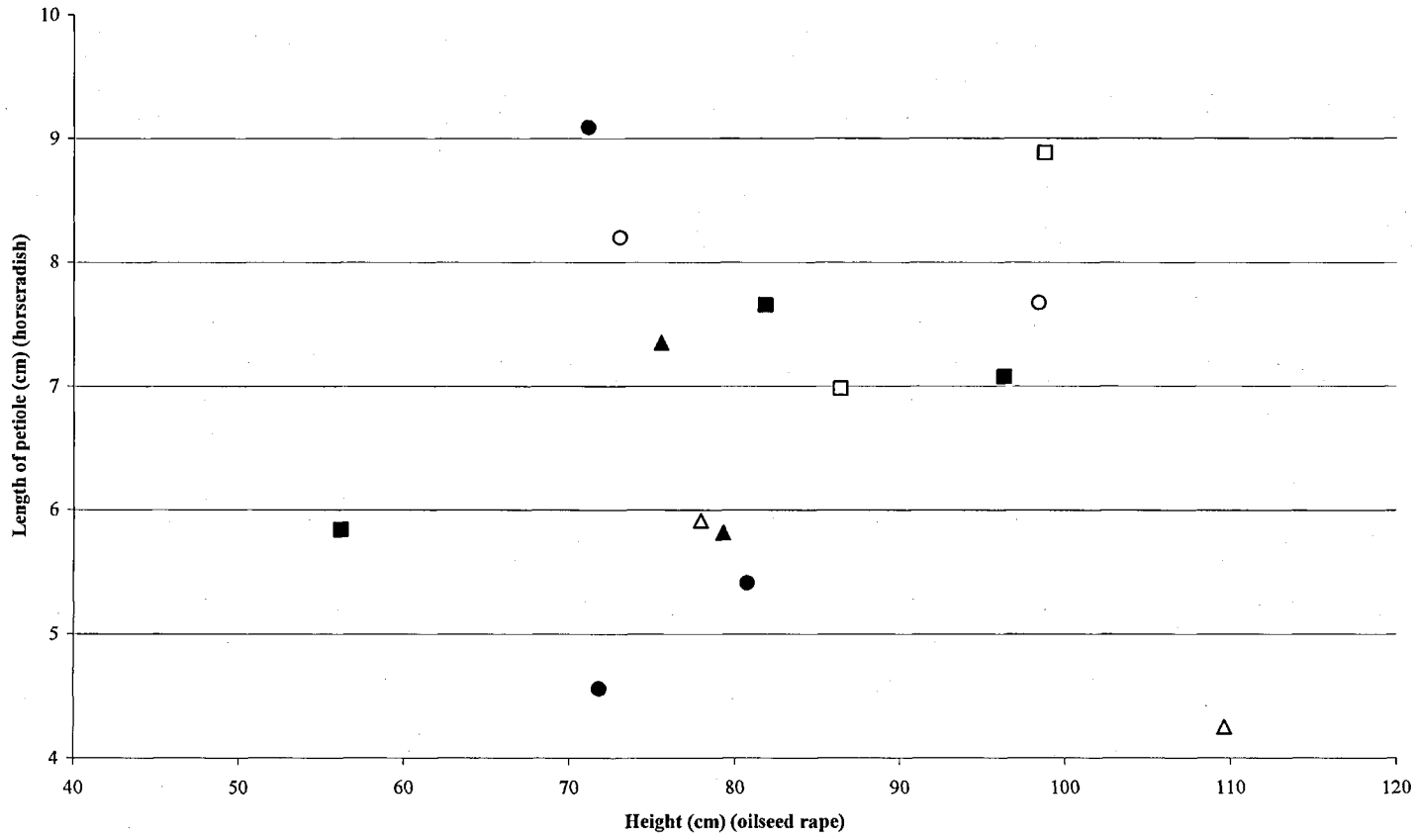

Figure 4.27. Results taken from infested soil experiment for only plants that grew. Score (oilseed rape) vs. Score (horseradish). ■ isolates from AFLP group α, ● isolates from AFLP group β, ▲ *V. dahliae* non-cruciferous isolates, □ control, ○ *V. albo-atrum* isolates, △ *V. dahliae* isolates from crucifers.

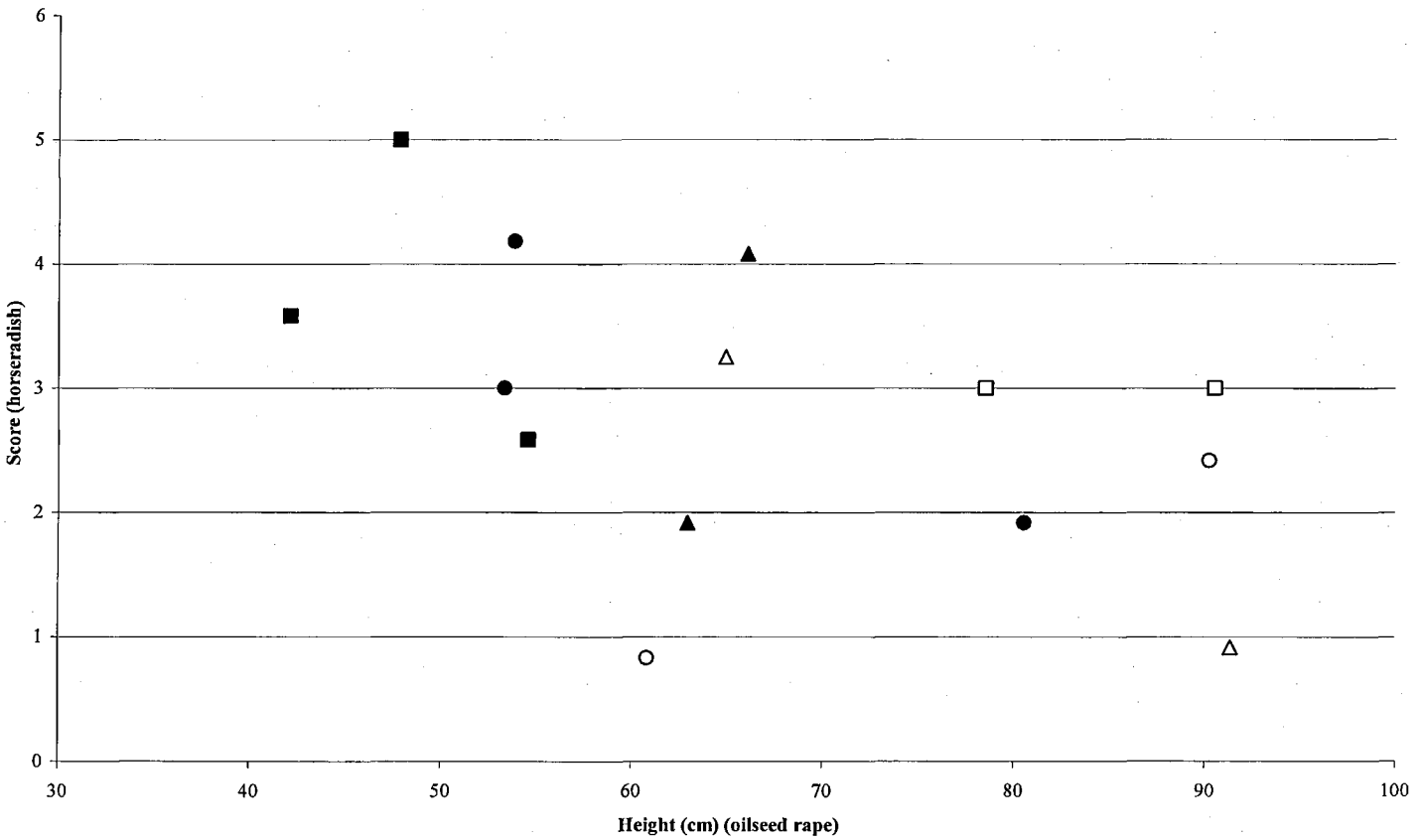

Figure 4.28. Results taken from infested soil experiment for all plants. Height (cm) (oilseed rape) vs. Score (horseradish). ■ isolates from AFLP group α, ● isolates from AFLP group β, ▲ *V. dahliae* non-cruciferous isolates, □ control, ○ *V. albo-atrum* isolates, △ *V. dahliae* isolates from crucifers.

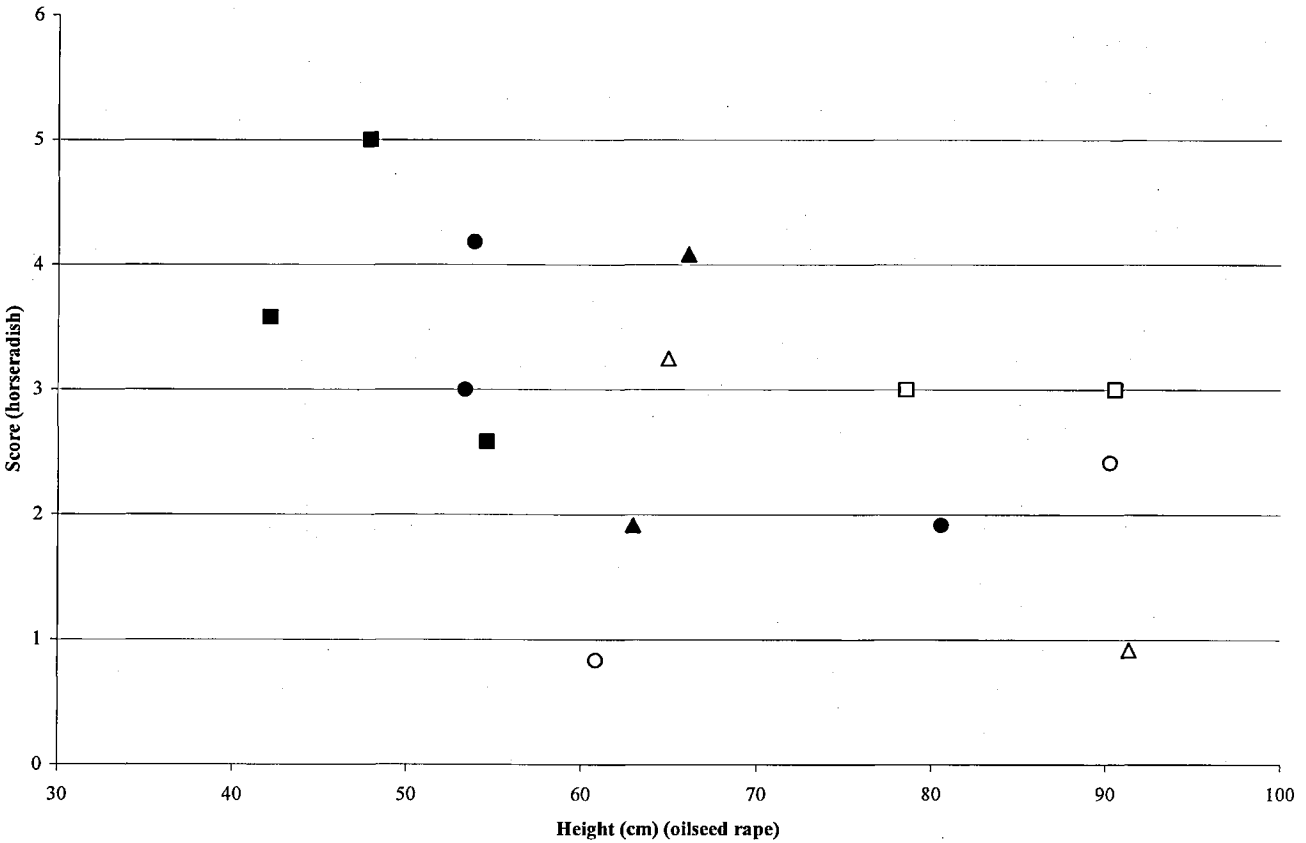

Figure 4.29. Results taken from infested soil experiment for only plants that grew. Height (cm) (oilseed rape) vs. Score (horseradish). ■ isolates from AFLP group α, ● isolates from AFLP group β, ▲ *V. dahliae* non-cruciferous isolates, □ control, ○ *V. albo-atrum* isolates, △ *V. dahliae* isolates from crucifers.

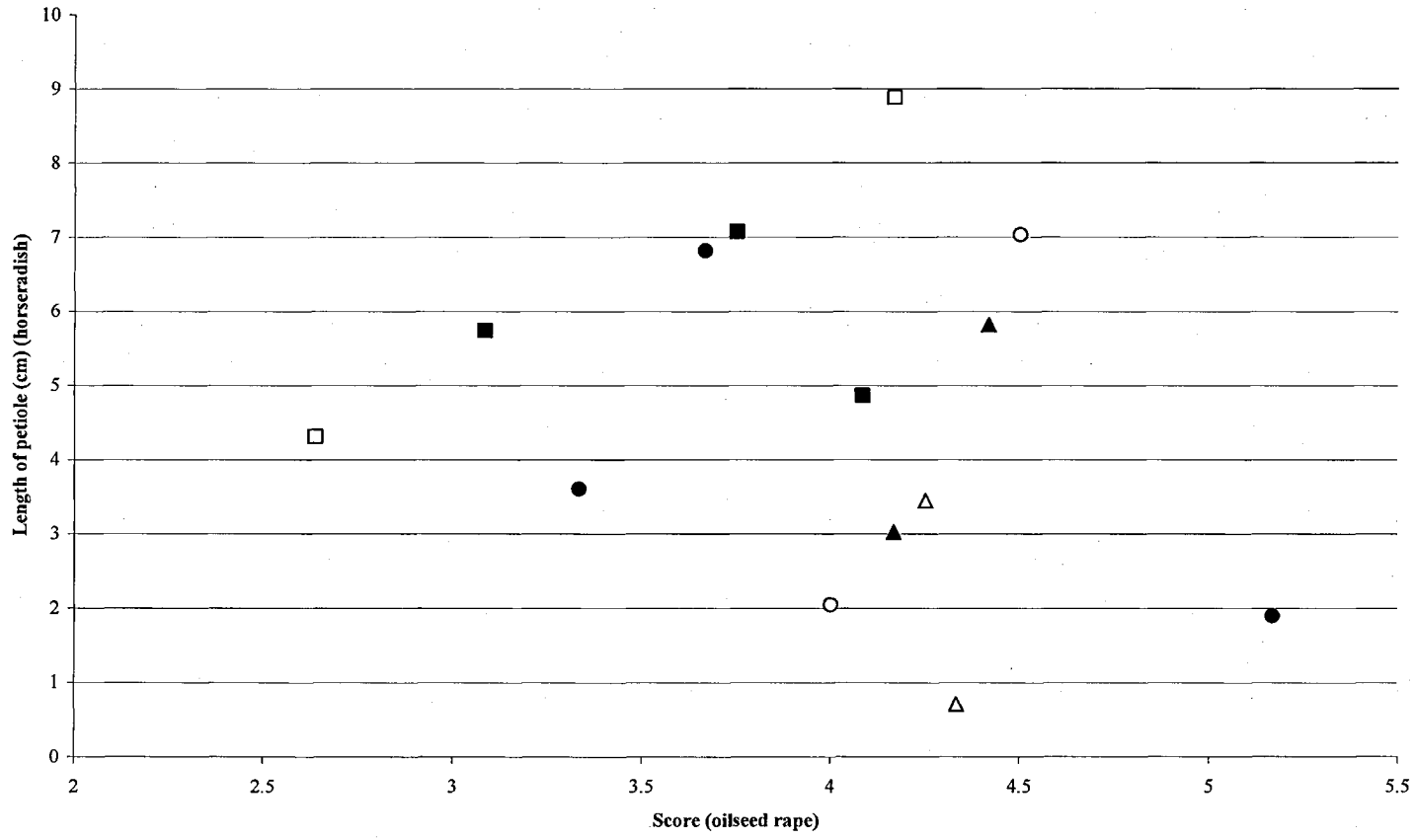

Figure 4.30. Results taken from infested soil experiment for all plants. Score (oilseed rape) vs. Length of petiole (cm) (horseradish). ■ isolates from AFLP group α, ● isolates from AFLP group β, ▲ *V. dahliae* non-cruciferous isolates, □ control, ○ *V. albo-atrum* isolates, △ *V. dahliae* isolates from crucifers.

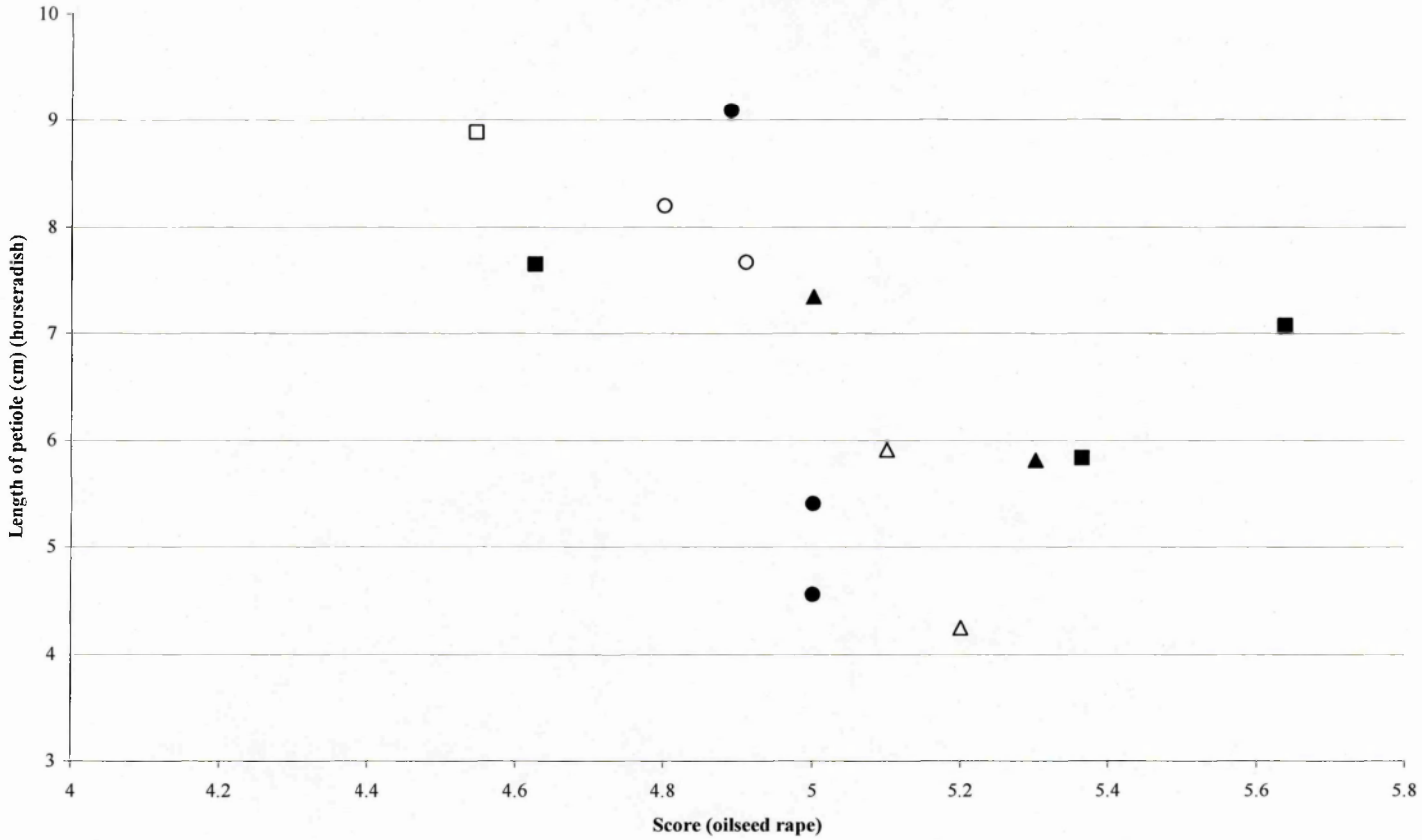

Figure 4.31. Results taken from infested soil experiment for only plants that grew. Score (oilseed rape) vs. Length of petiole (cm) (horseradish). ■ isolates from AFLP group α, ● isolates from AFLP group β, ▲ *V. dahliae* non-cruciferous isolates, □ control, ○ *V. albo-atrum* isolates, △ *V. dahliae* isolates from crucifers.

Crop		Comparison	Correlation coefficient	Figure
Oilseed rape	All Plants	Height V Scores	0.36	4.20
Oilseed rape	Plants that grew	Height V Scores	-0.09	4.21
Horseradish	All plants	Length* V Scores	0.59	4.22
Horseradish	Plants that grew	Length* V Scores	-0.71	4.23
Oilseed rape & Horseradish	All plants	Score V Scores	-0.29	4.24
Oilseed rape & Horseradish	Plants that grew	Score V Scores	0.16	4.25
Oilseed rape & Horseradish	All plants	Height V Length*	-0.08	4.26
Oilseed rape & Horseradish	Plants that grew	Height V Length*	-0.01	4.27
Oilseed rape & Horseradish	All plants	Height (OSR) V Score (HR)	-0.49	4.28
Oilseed rape & Horseradish	Plants that grew	Height (OSR) V Score (HR)	-0.05	4.29
Oilseed rape & Horseradish	All plants	Length* (HR) V Score (OSR)	-0.20	4.30
Oilseed rape & Horseradish	Plants that grew	Length* (HR) V Score (OSR)	-0.27	4.31

Table 4.4. Summary of correlation coefficients for all comparisons of infested soil test. Length* is length of horseradish petiole.HR is horseradish, OSr is oilseed rape.

One way ANOVA, (no blocking)

Horseradish; All Plants

Variate: Score	d.f.	s.s.	m.s.	v.r.	F. pr
Isolate	13	233.238	17.941	3.21	<.001
Residual	154	861.333	5.593		
Total	167	1094.571			

Tables of Means

Variate: Score

Horseradish Grand Mean 2.86

12087	**1974**	**84020**	**9802**	**9010**	**Md73**	**Md80**
4.08	2.42	5.00	1.92	3.58	4.50	0.92

001	**P14**	**STR1**	**Vd128**	**VdII**	**Plus**	**Without**
3.00	1.92	0.83	3.25	2.58	3.00	3.00

Standard errors of differences of means

Table	Isolate
Replicates	12
d.f.	154
s.e.d.	0.965

Table 4.5. ANOVA all plants horseradish score.

One way ANOVA, (no blocking)

Oilseed rape; All Plants

Variate: Score	d.f.	s.s.	m.s.	v.r.	F. pr
Isolate	13	62.935	4.841	1.07	0.385
Residual	154	693.917	4.506		
Total	167	756.851			

Tables of Means

Variate: Score

Oilseed rape Grand Mean 3.97

12087	**1974**	**84020**	**9802**	**9010**	**Md73**	**Md80**
4.42	4.50	3.75	5.17	4.08	3.33	4.33

001	**P14**	**STR1**	**Vd128**	**VdII**	**Plus**	**Without**
3.67	4.17	4.00	4.25	3.08	2.67	4.17

Standard errors of differences of means

Table	Isolate
Replicates	12
d.f.	154
s.e.d.	0.867

Table 4.6. ANOVA all plants oilseed rape score.

One way ANOVA, (no blocking)					
Horseradish; All Plants					
Variate: Length of petiole	d.f.	s.s.	m.s.	v.r.	F. pr
Isolate	13	842.06	64.77	3.98	<.001
Residual	154	2506.89	16.28		
Total	167	3348.95			

Tables of Means						
Variate: Length of petiole						
Horseradish Grand Mean 4.71						
12087	**1974**	**84020**	**9802**	**9010**	**Md73**	**Md80**
5.82	7.03	7.08	1.90	4.87	3.61	0.71
001	**P14**	**STR1**	**Vd128**	**VdII**	**Plus**	**Without**
6.82	3.68	2.05	3.45	5.74	4.32	8.88

Standard errors of differences of means	
Table	Isolate
Replicates	12
d.f.	154
s.e.d.	1.647

Table 4.7. ANOVA horseradish all plants length of petiole.

One way ANOVA, (no blocking)

Oilseed rape; All Plants

Variate: Height	d.f.	s.s.	m.s.	v.r.	F. pr
Isolate	13	42882	3299	1.53	0.112
Residual	154	331686	2154		
Total	167	374568			

Tables of Means

Variate: Height

Oilseed rape Grand Mean 67

12087	**1974**	**84020**	**9802**	**9010**	**Md73**	**Md80**
66.1	90.2	47.9	80.5	42.1	53.8	91.3

001	**P14**	**STR1**	**Vd128**	**VdII**	**Plus**	**Without**
53.3	62.9	60.8	64.9	54.5	79.2	90.5

Standard errors of differences of means

Table	Isolate
Replicates	12
d.f.	154
s.e.d.	18.95

Table 4.8. ANOVA oilseed rape all plant height.

4.4 Discussion

'Host-range specificity is a characteristic of an isolate from a particular host. In nature, a strong selection pressure is exerted on the field isolates, and only those isolates that are aggressive on a particular host can cause severe vascular discolouration and wilting' (Bhat and Subbarao, 1999).

The method of determining host-specificity of a particular isolate for a particular crop is affected by experimental procedures and this is an important consideration. Relatively little is known about the mechanisms behind infection of *Verticillium* in a host crop, and as such, certain methods of pathogenicity testing should only be employed if one accepts their limitations and do not rely too heavily on the relationships that may be inferred, then results can be useful to an extent.

It should be noted that in three of the four tests using the cut-root method the control plants, were similar in height to those infected, particularly those controls that had had their roots cut. This indicates the unreliability of this method of testing through the inherent nature of the invasive procedure. A major barrier to infection is of course the root cortex, and through its removal using this technique allows entry into the plant by the fungus through unnatural means. It also should be noted that as the fungus enters the plant as conidia this to adds to the artificial nature of this type of inoculation as conidia are normally only formed in the plants after a degree of post-infection mycelial growth.

However, accepting these limitations the cut-root method did give some distinction between amphihaploid and *V. dahliae* non-cruciferous isolates. The infested soil method

was intended to be a more 'natural' method of infection and thus the data gathered may reflect more accurately what is observed in the field. However it is important to realise that although the process of infection should be more 'natural', by introducing gross numbers of infective propagules, this method will also be to some extent 'unnatural'. It is known that the fungus on oat seed was viable through its growth on water agar, but whether once incorporated into the soil if this would remain the case is not known. Furthermore, it seems that incorporating uninoculated oat seed into the soil bore some inhibitory effects on the growth of both oilseed rape and horseradish, because the control left without oat seed seemed to grow better than those with seed added. Why this should be the case is not known, but it could be speculated that apart from the incorporation of oat seed into the soil affecting the soil chemistry through it subsequent degradation and possible affecting the growth of the plant that it may have acted as 'medium' for the growth of other soil-borne pathogens.

The pathology of *Verticillium* infection has meant that a rapid reliable bioassay has been lacking, and that methods on testing for resistance/pathogenicity are long, labour intensive and expensive. Most of the early evidence from the literature indicated that the differentiation of crucifer isolates from non-crucifer isolates in oilseed rape would be straightforward, it is only recently that crucifer isolates are being spoken of as not specific to the crucifers (Prof. K. V. Subbarao Personal communication). Results form the cut-root tests are similar to that of Karapapa *et al.* (1997), distinguishing long-spored oilseed rape isolates from *V. dahliae* non–cruciferous isolates. These authors did not include or have access to isolates of horseradish from Illinois (AFLP group β) and as such the apparent 'intermediate' pathogenicity of these isolates compared to the AFLP group α and *V. dahliae* non-cruciferous isolates would not have been recorded.

Whilst in this discussion these pathogenicity tests have been discussed in terms of distinguishing all the hybrids from haploid *V. dahliae* isolates, they were originally carried out to test the feasibility of distinguishing artificial hybrids from their 'parent' isolates. This was not possible in the infested soil experiments but in all the cut-root experiments the α group isolates could be distinguished from the non-crucifer *V. dahliae* haploid isolates. However whilst giving some stunting this was not true of all group β isolates. If it could be predicted that α group type isolates would be likely to be produced (possibly from the properties of the parent isolates) then a simple cut-root approach could be used. Distinguishing hybrids with β group pathogenicity would be more problematic. Why should only the long-spored isolates be taken from crucifers in the field? As little is relatively known about the epidemiology, or the mechanisms behind the infection of crucifers by long-spored isolates it is difficult to speculate as to why this should be the case if long-spored isolates are not host-specific.

4.5 Concluding remarks

Had the original premise of this PhD been undertaken, *i.e.* to generate artificial amphihaploids through protoplast fusion of *V. dahliae* and *V. albo-atrum* and to identify them by their supposed novel pathogenicity for crucifers, the experiment may not have been straightforward as the results presented here indicate that in these glasshouse experiments it is difficult to distinguish between *V. dahliae*, *V. albo-atrum* and some *Verticillium* isolates from crucifers. This is not to say that should the 'parents' of the long-spored amphihaploid isolates be identified, and artificial amphihaploids generated from them, that this would not be a useful course of action but as noted above the degree of distinction of any artificial hybrids from the parents cannot be predicted on the current

evidence. Therefore, it would be prudent to try to understand better the parentage of natural amphihaploid isolates as this may allow prediction of the pathogenic potential of novel hybrids from carefully chosen parents.

5 MOLECULAR IDENTIFICATION OF *VERTICILLIUM* SPECIES USING EXISTING MOLECULAR MARKERS

5.1 Introduction

5.1.1 Functional rRNA genes

In fungi, the nuclear rRNA genes are arranged as tandem repeats with more than 100 copies per genome. In *V. dahliae* the rRNA gene complex is *ca.* 7.2kb (Morton *et al.*, 1995a; Pramateftaki *et al.*, 2000) whereas most other filamentous fungi range from 7.7-12kb in size. Each rRNA gene repeat unit contains three rRNA genes: the small nuclear (18*S*-like) rRNA, the 5.8*S* rRNA and the large nuclear (28*S*-like) rRNA (Figure 5.1). The 5*S* rRNA genes are located elsewhere in the genome (Mukhamedov *et al.*, 1990) and will be discussed in Chapter 6.

5.1.2 Non-coding regions of the functional rRNA gene repeat unit

Within one gene repeat unit, the 5.8*S* rRNA gene is flanked by a bipartite internal transcribed spacer (ITS), the ITS1 and ITS2, which separates the 5.8*S* rRNA gene from the small and the large rRNA subunit genes, respectively. The intergenic spacer (IGS) separates the gene repeat units (Hillis and Dixon, 1991). The ITS1, ITS2 and IGS are removed during transcription and subsequent maturation of the functional rRNA molecules.

The functional rRNA genes evolve relatively slowly and are useful for comparing distantly related organisms. The non-coding regions (ITS and IGS) evolve at a faster rate and are useful for comparing isolates within species and species within a genus, as shown in *Verticillium* (Morton *et al.*, 1995a, b). Mutations in non-coding regions occur at a rate that approximates the rate of species emergence (Brasier *et al.*, 1999). Over time such mutations become fixed through crossing over and gene conversion, a process commonly referred to as concerted evolution (See 6.1.1 for fuller explanation). This process maintains the homogeneity of the gene cluster and spacer region. There is, however, a small but increasing number of reports that suggest that more than one form of the rRNA genes may exist within individuals of some species.

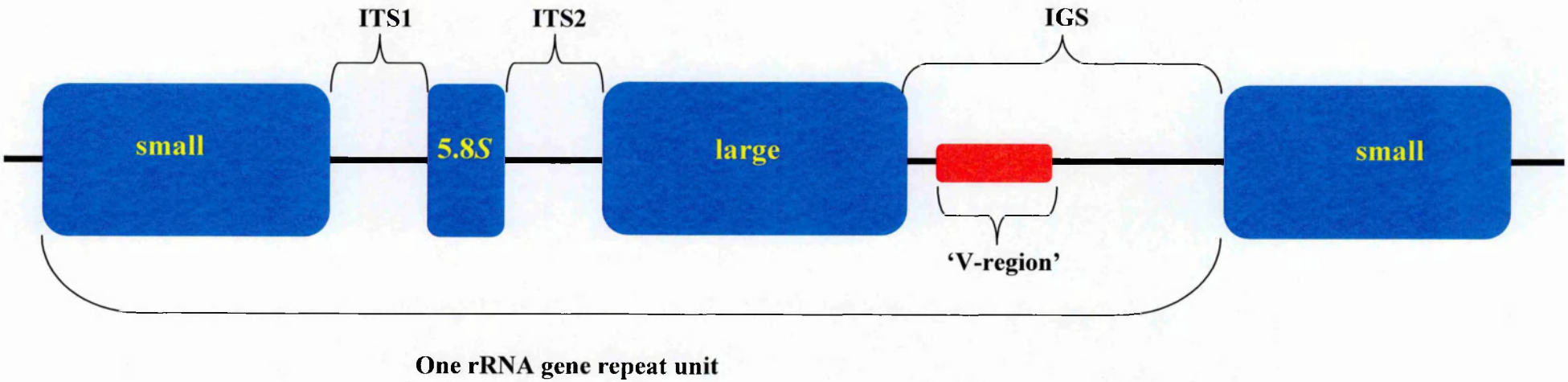

Figure 5.1. Schematic of the major rRNA gene repeat unit of *V. dahliae*. Shaded in■ are the functional rRNA genes; shaded in■ is the 'V-region' (Collins *et al.*, 2003)

5.1.2.1 ITS

In *Phytophthora* it has been suggested that the occurrence of different ITS types is due to interspecific hybridisation or gene duplication (Brasier *et al.*, 1999). Such additivity has also been demonstrated in the rRNA genes of allopolyploid species hybrids in plants, and as previously mentioned following a hybridisation event, bidirectional concerted evolution generally leads to homogenisation and the retention of only one of the parental ITS types. In the absence of either crossing over or chromosomal loss, parental rRNA gene arrays on homologous chromosomes would be expected to remain discrete and in their parental form.

In *Fusarium*, some species that have arisen through interspecific hybridisation have been identified by the presence of different rRNA types. The most abundant rRNA ITS type is referred to as the major ITS type and the less frequently occurring repeat is referred to as the minor ITS type (O'Donnell and Cigelnik, 1997; O'Donnell *et al.*, 1998). It should be highlighted, however, that these hybrids have the same genetic state as that of the presumed parent species (*i.e.* they are haploid), unlike here with *Verticillium* amphihaploid isolates.

Collins *et al.* (2003) utilised the non-coding regions of the rRNA genes to investigate the relationships between isolates at the sub-genus level at a single locus. By correlating ITS sequence variants (Figure 5.2) with spore length/DNA content, *Verticillium* crucifer isolates were separated into four groups. The first group is represented by *Verticillium* isolates from cruciferous crops in Europe, Japan, Russia and USA. They are long-spored and have a high DNA content and are presumed hybrids. Additionally, they possess ITS

sequences that are similar to, but distinct from, *V. albo-atrum* (L) (Collins *et al.,* 2003; Morton *et al.*, 1995b). It was proposed by Karapapa *et al.* (1997) that a post-hybridisation event involving a *V. albo-atrum* (L) and subsequent genetic drift lead to sequence divergence. In addition, these *Verticillium* crucifer isolates contained homogenous rRNA gene repeats that may have resulted from either i) unequal crossing over between parental chromosomes following hybridisation leading to homogenisation and the emergence of only one of the parental ITS types or alternatively ii) loss of relevant parental chromosome(s) following hybridisation. The latter would explain why these isolates share more similarity to *V. albo-atrum* than *V. dahliae* at the ITS level when RAPD analysis showed them to be more *V. dahliae*-like (Karapapa *et al.*, 1997).

The second group, according to ITS, comprises the original *V. dahliae* var. *longisporum* isolate (Stark, 1961). It is characteristically long-spored/high DNA and has a *V. dahliae*-like ITS sequence that is not identical with any other seen. However, rather than the involvement of a *V. dahliae* isolates with a novel ITS sequence, it is more probable that genetic drift lead to sequence divergence over time. Furthermore, the isolate contains homogenous rRNA gene repeats implying that within a few generations selection is conferred at the genetic level with regard to the parental ITS sequences.

The third group found in this analysis is represented by a single oilseed rape isolate from Germany (Md73). It possessed the long-spored/high DNA content characteristics and its ITS sequence was identical to the majority of *V. dahliae* isolates. It was proposed by Collins *et al.* (2003) that some isolates might maintain a major (i.e. comprising the majority of rRNA repeats) and minor (i.e. occurring only in a small proportion of rRNA repeats) forms of the ITS sequence. But this was the only isolate for which strong evidence

of two rRNA types being retained was presented (Collins *et al.*, 2003). This is analogous to *Fusarium* and *Phytophthora* where it had been suggested that different ITS types are apparent through interspecific hybridisation or gene duplication. The minor ITS sequence that was *V. albo-atrum*-like, akin to that of the majority of *Verticillium* crucifer isolates found in group 1. There are potentially many explanations as to why rRNA gene non-coding region polymorphisms persist long-term following hybridisation i) their chromosomal locations may be centromeric rather than telomeric ii) the sequences may have diverged beyond the point that interchromosomal conversion can occur iii) concerted evolution may be more efficient within a chromosomal locus than between repeats dispersed on nonhomologous chromosomes (O'Donnell and Cigelnik, 1997). It is not clear why Md73 should be unusual in this respect.

The final group of *Verticillium* crucifer isolates according to ITS sequence variants is represented by short-spored crucifer isolates and one long-spored isolate. These isolates possessed a major ITS type identical to that of *V. dahliae*. However, a minor ITS type the same as to that seen in group 1 was also apparently retained by the short-spored isolates. It is possible that these isolates derived from a long-spored/high DNA content isolate that arose following hybridisation with a *V. dahliae* of this ITS type and a *V. albo-atrum*-like isolate with a novel ITS type.

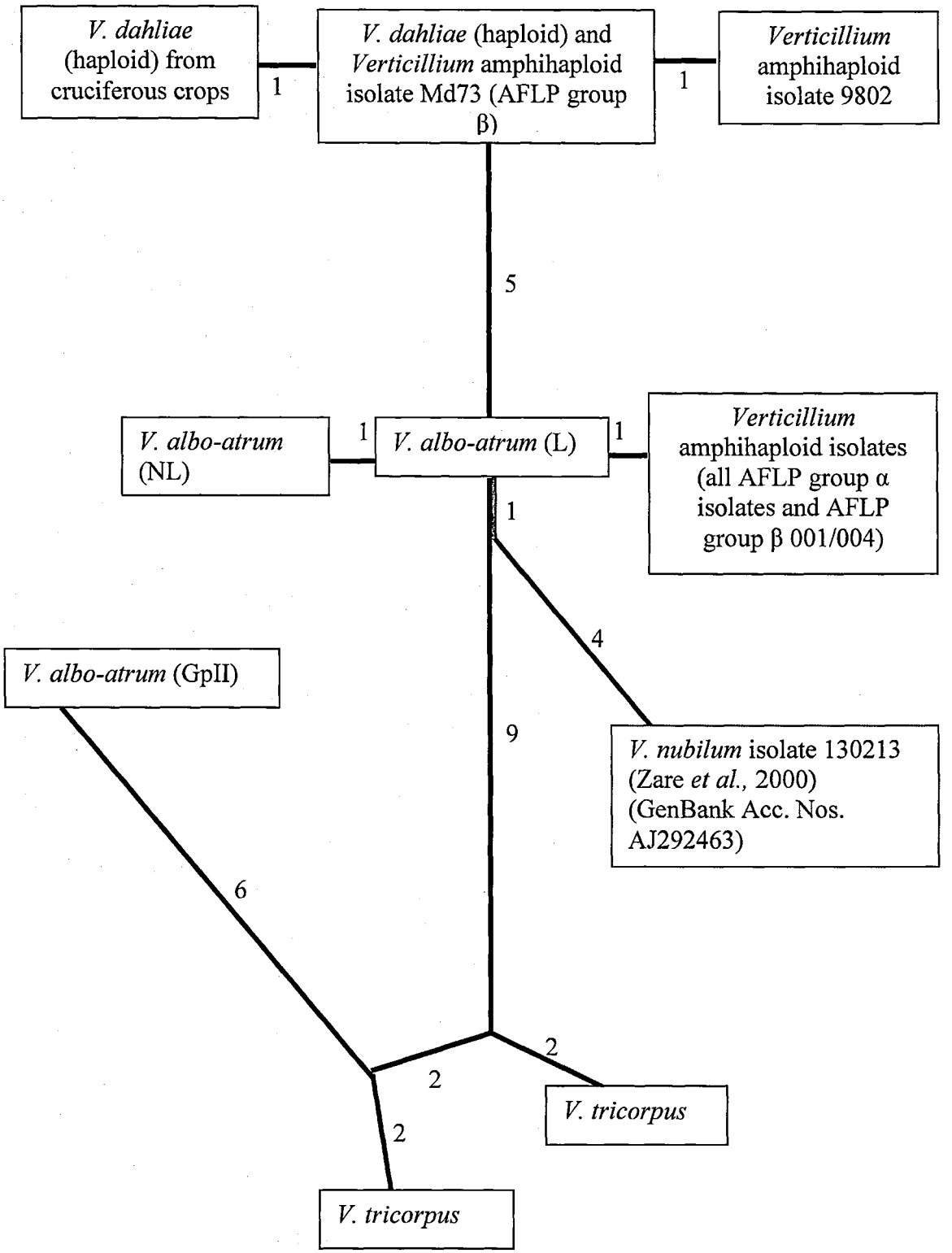

Figure 5.2. Sequence variants of rRNA gene ITS of main plant pathogenic *Verticillium* species adapted from Morton *et al.* (1995b) and Collins (2002). Numbers against lines represent number of nucleotide differences. Lines and words in blue indicates sequence taken from GenBank.

5.1.2.2 IGS

Molecular analysis of the IGS sub-repeats showed that although sequences differed between isolates, the structure was common across all isolates. A 320 bp non-repetitive sequence was elucidated from this analysis that had not been described previously. The implications of this sequence and its potential will be discussed later. The overall structure of the sub-repeats correlated with groups given by ITS sequence data apart from that of the *V. dahliae* var. *longisporum* isolates and a German oilseed rape isolate. This latter isolate (Md73) retained a minor IGS sub-repeat sequence (in addition retained a minor ITS *V. albo-atrum*-like sequence as mentioned previously).

Undertaken within the same set of studies, Collins *et al.* (2003) analysed sub-repeats within the IGS of the rRNA gene tandem array. Two 18 bp imperfect repeats were found in all isolates 320 bp downstream from an *Eco*RI restriction endonuclease site occurring at the end of the sub-repeats. This 320 bp non-repetitive region between the main region of sub-repeats and the two 18 bp imperfect repeats is termed the V-region. Within the 320 bp V-region, Collins *et al.* (2003) found 98 polymorphic positions, the majority of which were single base changes (87/98) with the remaining 11 as double base changes. These polymorphisms were used to generate an unrooted tree to illustrate the interrelationships between these isolates at this locus (Figure 5.3).

For the haploid *V. dahliae* non-cruciferous isolates tested (Md71 and Md124) the sequences were found to be identical to those previously published (Morton *et al.*, 1995b); for the haploid *V. dahliae* cruciferous isolates tested (Md80 and Vd128) they were found to

be identical and separated from other haploid *V. dahliae* by 4 bp. *Verticillium* amphihaploid isolates from AFLP group β (9802 and Md73), that were *V. dahliae*-like by ITS, were 1 bp and 3 bp different from the haploid *V. dahliae* non-cruciferous and cruciferous isolates respectively. For the remaining amphihaploid isolates, that according to ITS were all *V. albo-atrum*-like, the V-region sequences were similar to each other and very different from any isolate with *V. dahliae*-like ITS or any of the *V. albo-atrum* isolates tested. Two isolates from horseradish, 001 and 004 (*Verticillium* amphihaploid AFLP group β) were identical to each other and differed from majority of amphihaploid isolates (AFLP group α) by 2 bp (Figure 5.3).

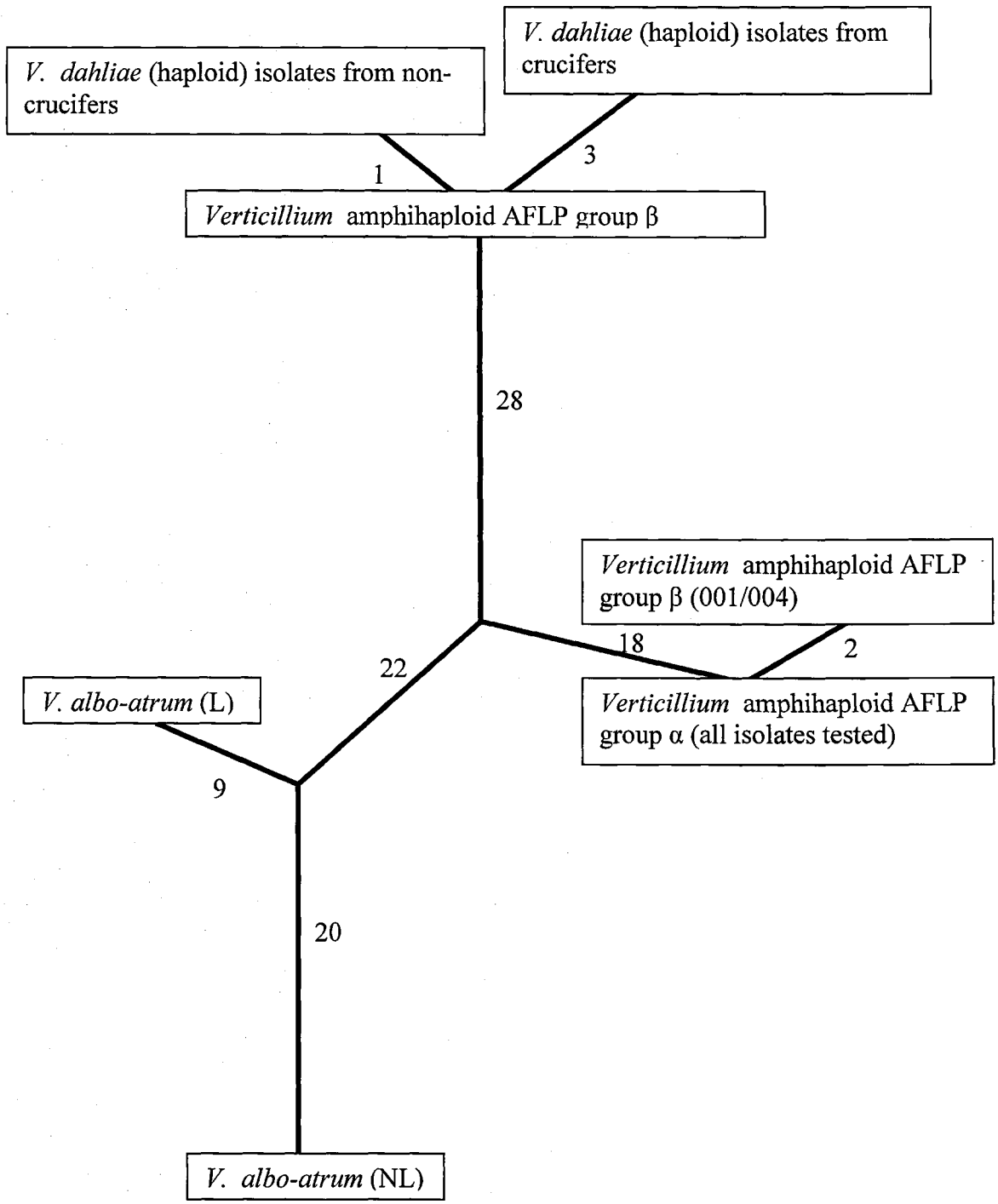

Figure 5.3. Sequence variants of 'V-region' of main plant pathogenic *Verticillium* species adapted from Collins *et al.* (2003). Numbers against lines represent number of nucleotide differences.

5.1.3 Defoliating and non-defoliating pathotypes of *V. dahliae*

Isolates of *V. dahliae* from cotton and olive are classified according to their virulence, as defoliating (D) and non-defoliating (ND) pathotypes. However, to discern between these two pathotypes by traditional methods is time consuming and does not indicate the genetic diversity among or within populations. Through RAPD analysis, Pérez-Artés *et al.* (2000) determined that there were two clear populations amongst *V. dahliae* isolates from cotton and olive that correlated according to D and ND pathotypes. Amongst D isolates using RAPD data there was no variation but ND isolates were found to be variable.

From this RAPD analysis it was possible to derive pathotype-specific PCR primers which have subsequently been used in the detection of *V. dahliae* D (Mercado-Blanco *et al.*, 2002) and ND (Mercado-Blanco *et al.*, 2001) pathotypes *in planta*. These molecular markers may prove useful in the detection of the origins of outbreaks or could be used as another marker that may identify a possible parent in an interspecific hybridisation. For example, the distinctness of the D and ND pathotypes both molecularly and in VCG assignment suggests that the D isolates are not variants derived from the native variable *V. dahliae* population. Additionally, as the defoliating disease was reported in Spain more recently than the mild disease it can be assumed that this is the alien introduction, this theory is by the fact that isolates from the homogenous D population was identical to isolates from California and China (Mercado-Blanco *et al.*, 2002). The D and ND primers have made apparently interesting sub-divisions of haploid *V. dahliae* isolates, and it is hoped that when these primers are tested on amphihaploid isolates that evidence should be presented with regard to their parental origins.

5.1.4 New Diseases

In modern pathology, there is a constant stream of diseases in 'new' host crops and 'new' diseases and it is necessary to be able set apart these from those already established. At Warwick HRI, we as a research group have gained a reputation for the molecular identification of plant pathogenic *Verticillium* species, particularly the main pathogens, *V. dahliae*, *V. albo-atrum* and the amphihaploid isolates, and are requested by others to carry out limited molecular characterisation of these isolates. When apparently unusual isolates become evident to other groups in their studies, or to us through their publications, we are able to test these using existing molecular methods to study new disease outbreaks.

5.1.5 Aims and Objectives

- Attempt to identify possible parents of *Verticillium* amphihaploids, using existing molecular markers, from new and apparently unusual isolates.
- Molecular identification of possible amphihaploid isolates.
- Provide initial molecular identification of *Verticillium* species.
- Look for the presence of defoliating and non-defoliating pathotype specific markers in amphihaploid isolates.

5.2 Methods

5.2.1 PCR and restriction endonuclease analysis of the ITS

DNA extraction and PCR components are as described in Chapter 2. ITS sequences were amplified using primers ITS4 and ITS5 (Table 2.2). The reaction mixture was subjected to an initial denaturation (93°C/2min) and an annealing step (54°C/15s) and then cycled 40 times (72°C/30s, 93°C/15s, 54°C/15s) before a final 72°C/10min.

Where deemed appropriate PCR products of ITS4/ITS5 were digested by restriction endonucleases for analysis. 2-5μl of PCR products were digested according to the enzyme manufacturer's instructions for 150 minutes in a total volume of 10μl, and DNA fragments from digestions were separated by electrophoresis on appropriate agarose gels in 1xTBE. Staining was with ethidium bromide.

5.2.2 PCR of V-region

DNA extraction and PCR components are as described in Chapter 2. The 'V-region' of the IGS sequence of the rRNA gene repeats was amplified using primers 723 and 727 (Table 2.2) (Collins *et al.*, 2003). Thermal cycling conditions were as above except that the annealing temperature was 58°C.

5.2.3 PCR using primers for defoliating and non defoliating pathotypes

DNA extraction and PCR components are as described in Chapter 2. Two sets of primers described as being able to differentiate between the defoliating and non-defoliating pathotypes and were tested on a range of cruciferous and non-cruciferous isolates,

including known defoliating and non-defoliating isolates of *V. dahliae* as positive controls. Primers 858 (INTD2F) and 859 (INTD2R) gave products for the defoliating pathotype, and primers 860 (INTND2F) and 861 (INTND2R) for the non–defoliating pathotype (Mercado-Blanco *et al.*, 2001; 2002) (Table 2.2). PCR conditions were as standard with an annealing temperature of 64°C.

5.2.4 Sequence analysis

PCR products were sequenced directly by the dideoxyribonucleotide chain termination method using Big Dye chemistry on an automated sequencer (ABI) at Warwick HRI, UK. Sequences were edited and aligned using EditSeq and MegAlign, part of Lasergene v5.07 (DNAStar Inc. USA).

Isolate	Host	Origin	Purported species	Source
Hr4	*Armoracia rusticana*	Illinois, USA	*V. dahliae*[1]	WC
Hr8	*A. rusticana*	Illinois, USA	*V. dahliae*[1]	WC
Hr14	*A. rusticana*	Illinois, USA	*V. dahliae*[1]	WC
Hr24	*A. rusticana*	Illinois, USA	*V. dahliae*[1]	WC
Hr28	*A. rusticana*	Illinois, USA	*V. dahliae*[1]	WC
Wvl2	*Brassica oleracea* ssp. *botrytis*	Belgium	*V. dahliae*[1]	JD
K1	*B. oleracea* ssp. *botrytis*	Belgium	*V. dahliae*[1]	JD
O1	*B. oleracea* ssp. *botrytis*	Belgium	*V. dahliae*[1]	JD
P4	*B. oleracea* ssp. *botrytis*	Belgium	*V. dahliae*[1]	JD
S3	*B. oleracea* ssp. *botrytis*	Belgium	*V. dahliae*[1]	JD
Be1	*B. oleracea* ssp. *botrytis*	Holland	*V. dahliae*[1]	JD
Pf1	*B. oleracea* ssp. *botrytis*	Germany	*V. dahliae*[1]	JD
Po32nitM	*B. napus* ssp. *oleifera*	Poland	*V. dahliae*[1]	MRG
Po180nit1	*B. napus* ssp. *oleifera*	Poland	*V. dahliae*[1]	MRG
NR1-fito	*B. napus* ssp. *oleifera*	Europe	*V. dahliae*[1]	DG
NR2-fito	*B. napus* ssp. *oleifera*	Europe	*V. dahliae*[1]	DG
PE6971	*Pisum*	Canada	*V. albo-atrum*	HWP
PE6972	*Pisum*	Canada	*V. albo-atrum*	HWP
PE6973	*Pisum*	Canada	*V. albo-atrum*	HWP
P1855	Spinach seed	Canada	*V. albo-atrum*	HWP
P1856	*Solanum tuberosum*	Canada	*V. albo-atrum*	HWP
P2934	Soil under *S. tuberosum*	Canada	*V. albo-atrum*	HWP
P2935	Soil under *S. tuberosum*	Canada	*V. albo-atrum*	HWP
VA104	Soil under *S. tuberosum*	Prince Edward Island	*V. albo-atrum* (II)	HWP
VA175A	*S. tuberosum*	Holland	*V. albo-atrum* (II)	HWP
PE5901	unknown	Prince Edward Island	*V. albo-atrum* (II)	HWP
PE6902	unknown	Unknown	*V. dahliae*	HWP
PE69212A	unknown	Unknown	*V. dahliae*	HWP
Vd.LH.25	unknown	Israel	*V. tricorpus*	LT
Ir1	*Medicago sativa*	Markazi, Iran	*V. albo-atrum* (L)	MG
Ir2	*M. sativa*	Markazi,Iran	*V. albo-atrum* (L)	MG
Ir3	*M. sativa*	Markazi,Iran	*V. albo-atrum* (L)	MG
130213	unknown	Scotland, UK	*V. nubilum**	IMI
964.95	Soil	Papua New Guinea	*V. nubilum*	CVS
8266	Soil	Germany	*V. nubilum*	BCCM
225818	unknown	unknown	*V. theobromae*	RC
29778	*Musa* sp.	unknown	*V. theobromae*	BCCM
15114	Soil	Canada	*V. nigrescens*	BCCM
1953	***Humulus lupulus***	**England**	***V. albo-atrum***	**WHRI**
1974	***H. lupulus***	**England**	***V. albo-atrum***	**WHRI**
STR1	***M. sativa***	**Canada**	***V. albo-atrum* (L)**	**KB**
STR3	***M. sativa***	**Canada**	***V/ albo-atrum* (L)**	**KB**
Md71	***Matricaria chamomilla***	**Germany**	***V. dahliae***	**HP**

12087	***Fragaria anannassa***	**Germany**	***V. dahliae***	**DH**
P14	***Lycopersicon esculentum***	**Brazil**	***V. dahliae***	**AS**
V-017	***Cynara scolymus***	**Spain**	***V. dahliae*(D)**	**RJD**
1381	***Gossypium spp.***	**Spain**	***V. dahliae*(D)**	**RJD**
1771	***Gossypium spp.***	**Spain**	***V. dahliae*(D)**	**RJD**
Md80	***B. napus* ssp. *oleifera***	**Germany**	***V. dahliae*[2]**	**HP**
Vd128	***B. oleracea* ssp. *botrytis***	**Germany**	***V. dahliae*[2]**	**MC**
90-03	***B. oleracea* ssp. *botrytis***	**California, USA**	**α**	**SK**
VdII	***B. napus* ssp. *oleifera***	**Germany**	**α**	**BH**
617	***B. napus* ssp. *oleifera***	**France**	**α**	**HB**
334	***B. napus* ssp. *oleifera***	**Germany**	**α**	**HB**
001	***A. rusticana***	**Illinois, USA**	**β**	**DE**
004	***A. rusticana***	**Illinois, USA**	**β**	**DE**
9802	***A. rusticana***	**Germany**	**β**	**BCCM**
Md73	***B. napus* ssp. *oleifera***	**Germany**	**β**	**HP**

Table 5.1. Isolates of plant pathogenic *Verticillium* used for these studies. Source: WC, Dr W. Chen; JD, Dr J. Debode; MRG, Dr M. Rataj-Guranowska; DG, Dr D. Gkilpathi; HWP, Dr H. W. Platt; LT, Dr L. Tsror; MG, Dr M. Ghalander; RC Dr R. Cooper; WHRI, Warwick HRI collection; KB, Dr K. Broersma; HP, Dr H. Prillinger; DH, Dr D. Harris; AS, Dr. A Soares; Prof. R. Jimenez-Diaz; MC, Prof. M. Cirulli; SK, Dr S. Koike; BH, Dr B. Holtschulte; DE, Prof. D. Eastburn; [1] isolates of *V. dahliae* from cruciferous crops that are likely to be amphihaploid; [2] haploid isolates of *V. dahliae* from cruciferous crops as described by Collins *et al.* (2003).* supplied to us as *caveat emptor* with regard to its species identity as suppliers regarded it as divergent from the species. IMI, BCCM, BCCM™/MUCL; CVS, Centraalbureau voor Schimmelcultures. Isolates in bold were received and made part of the Warwick HRI collection prior to these studies.

5.3 Results

5.3.1 Identification of 'new' *Verticillium* isolates from cruciferous crops

5.3.1.1 Isolates of *V. dahliae* from horseradish in Illinois

Amplification by PCR using primers ITS 4 and ITS5 yielded products of approximately 600 bp (Figure 5.4) from all isolates (Hr4, Hr8, Hr14, Hr24, and Hr28). Isolates were digested with enzymes *Tsp*EI, *Alu*I, and *Hae*II for restriction endonuclease analysis. By RE analysis, isolates Hr4 and Hr28 were shown to be *V. albo-atrum*-like whereas isolates Hr8, Hr14, and Hr24 were shown to be *V. dahliae*-like (Table 5.2).

Sequence analysis showed that according to the ITS, isolates HR4 and HR28 are identical with previously described horseradish isolates 001 and 004 (amphihaploid AFLP group β), whereas isolates HR8, HR14 and HR24 were found to be identical with haploid *V. dahliae* isolates Md80 and Vd128 except for one unresolved base in HR8 (Figure 5.5).

5.3.1.2 Isolates of *V. dahliae* from oilseed rape from Northern Europe

Amplification by PCR using primers ITS4 and ITS5 yielded products of approximately 600 bp from all isolates (Po32*nit*M, Po180*nit*1, NR1-fito, NR2-fito). Isolates were digested for restriction endonuclease analysis using enzymes as described as above. By RE analysis isolates Po32*nit*M and Po180*nit*1 were shown to be *V. dahliae*-like, and NR1-fito and NR2-fito were shown to be *V. albo-atrum*-like.

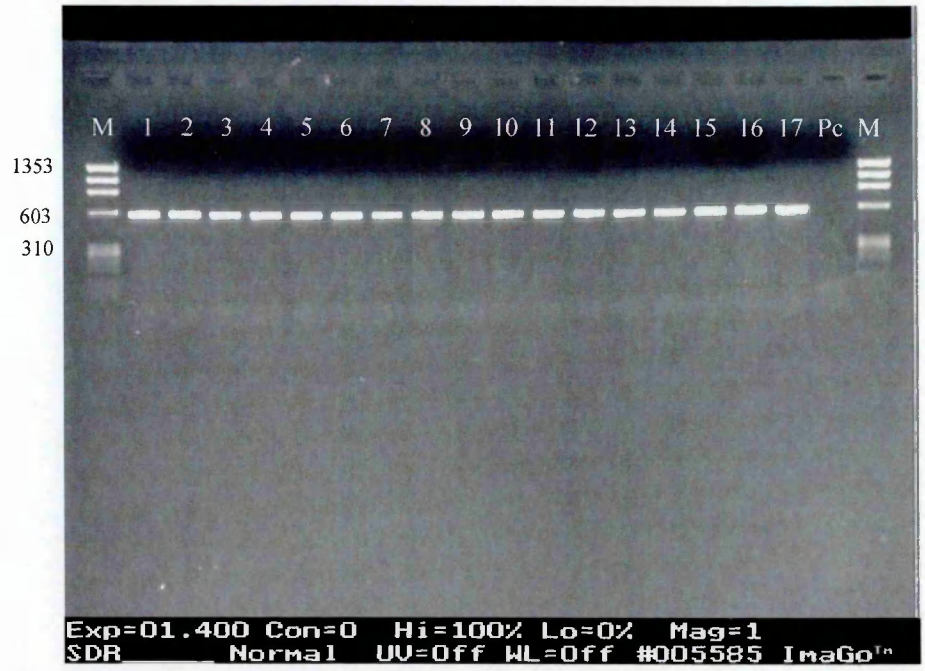

Figure 5.4. Agarose gel electrophoresis of ITS1-5.8*S*-ITS2 rRNA gene products from amplification with primers ITS4 and ITS5. Lanes 1-5 haploid *V. dahliae* (1: 12087, 2: Md71, 3: P14, 4: Md80, 5: Vd128), lanes 6-8 *Verticillium* amphihaploid AFLP group α isolates (6:9010, 7: VdII, 8: 84020), lanes 9-12 *Verticillium* amphihaploid AFLP group β isolates (9: 9802, 10: Md73, 11: 001, 12: 004), lanes 13-14 *V. albo-atrum* (L) (13: STR1, 14: STR3), lanes 15 *V. albo-atrum* (NL) (15: VA1 16: 1974), lane 17 *V. nubilum*: 130213 Pc: primer control, M: molecular marker.

Isolate	Enzyme			Species?
	***Tsp*EI**	***Alu*I**	***Hae*II**	
HR4	*V. albo-atrum*	*V. dahliae/ V. albo-atrum*	*V. albo-atrum*	*V. albo-atrum*[1]
HR8	*V. dahliae*	*V. dahliae/ V. albo-atrum*	*V. dahliae*	*V. dahliae*
HR14	*V. dahliae*	*V. dahliae/ V. albo-atrum*	*V. dahliae*	*V. dahliae*
HR24	*V. dahliae*	*V. dahliae/ V. albo-atrum*	*V. dahliae*	*V. dahliae*
HR28	*V. albo-atrum*	*V. dahliae/ V. albo-atrum*	*V. albo-atrum*	*V. albo-atrum*[1]
Po32*nit*M	*V. dahliae*	*V. dahliae/ V. albo-atrum*	*V. dahliae*	*V. dahliae*
Po180*nit*1	*V. dahliae*	*V. dahliae/ V. albo-atrum*	*V. dahliae*	*V. dahliae*
NR1-fito	*V. albo-atrum*	*V. dahliae/V. albo-atrum*	*V. albo-atrum*	*V. albo-atrum*[1]
NR2-fito	*V. albo-atrum*	*V. dahliae/V. albo-atrum*	*V. albo-atrum*	*V. albo-atrum*[1]
P1855	*V. dahliae**	NR	NR	*V. dahliae**
P1856	*V. albo-atrum*	*V. dahliae/ V. albo-atrum*	NT	*V. albo-atrum*
P2934	*V. albo-atrum*	*V. dahliae/ V. albo-atrum*	NT	*V. albo-atrum*
P2935	*V. albo-atrum*	*V. dahliae/ V. albo-atrum*	NT	*V. albo-atrum*
VA104	*V. albo-atrum* (II)	NT	NT	*V. albo-atrum* (II)
VA175A	*V. albo-atrum* (II)	NT	NT	*V. albo-atrum* (II)
PE5901	*V. albo-atrum* (II)	NT	NT	*V. albo-atrum* (II)
PE6902	*V. dahliae*	*V. dahliae/ V. albo-atrum*	NT	*V. dahliae*
PE69212A	*V. dahliae*	*V. dahliae/ V. albo-atrum*	NT	*V. dahliae*
Vd.LH.25	*V. albo-atrum/ V. tricorpus*	*V. tricorpus/V. albo-atrum* (II)	*V. dahliae/V. tricorpus/V. albo-atrum* (II)	*V. tricorpus*

Table 5.2. Summarised results of RE analysis.* indicates a *V. dahliae*-like pattern. [1]indicates *V. albo-atrum* pattern from a crucifer isolate, NR indicates no result and NT indicates that it was not tested.

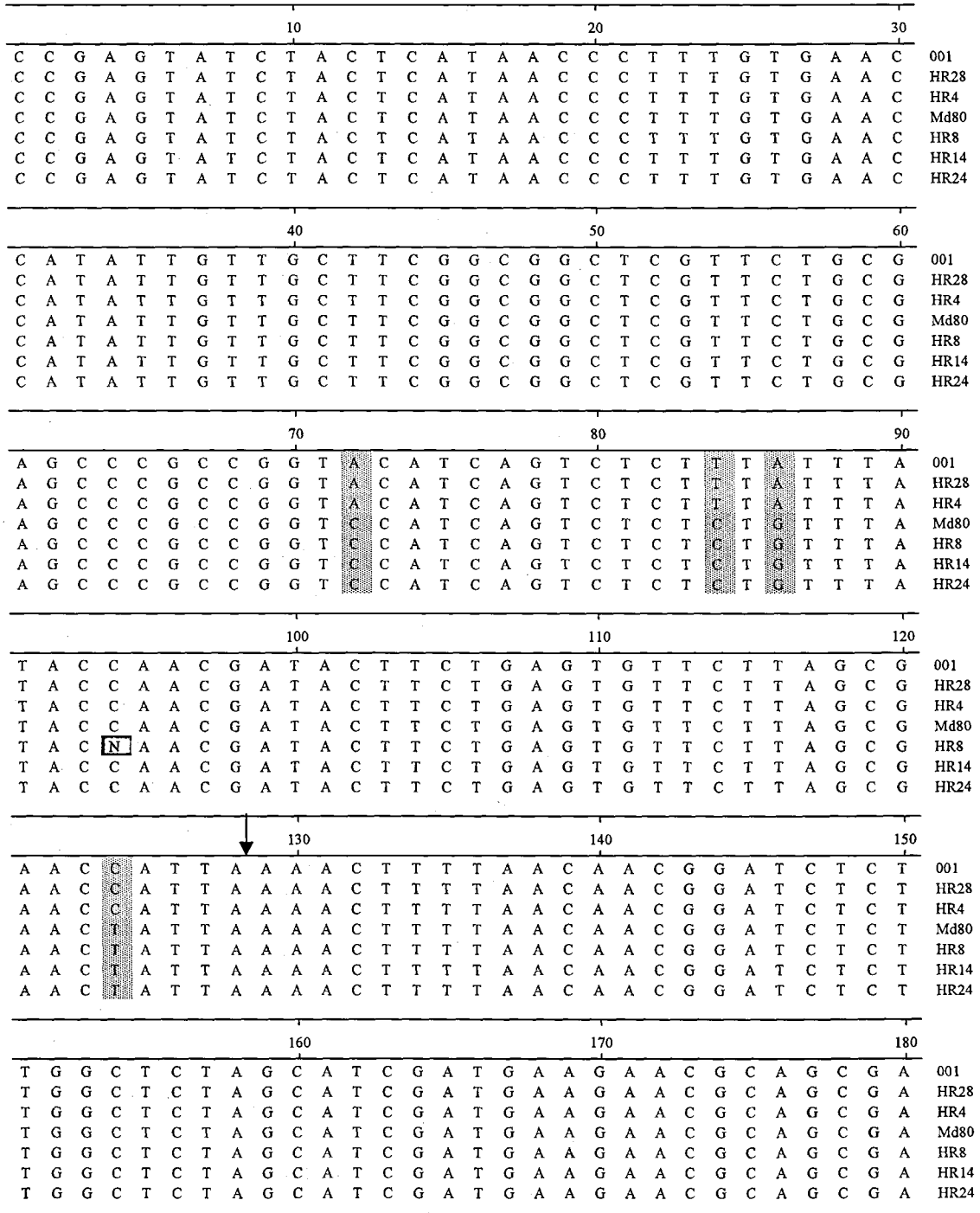

Figure 5.5. Sequence alignment of ITS1-5.8S-ITS2 of isolates of *Verticillium* isolates from horseradish (HR1, 4, 8,14, 24, 28), with previously characterised *Verticillium* isolates from horseradish (001 AFLP group β) and haploid *V. dahliae* (Md80). Shaded areas indicate sequence differences. ITS1:1-128; 5.8*S*: 129-285; ITS2: 286-452.'N' shaded in red is an unresolved base. Junctions between regions is indicated by an →. Continued.

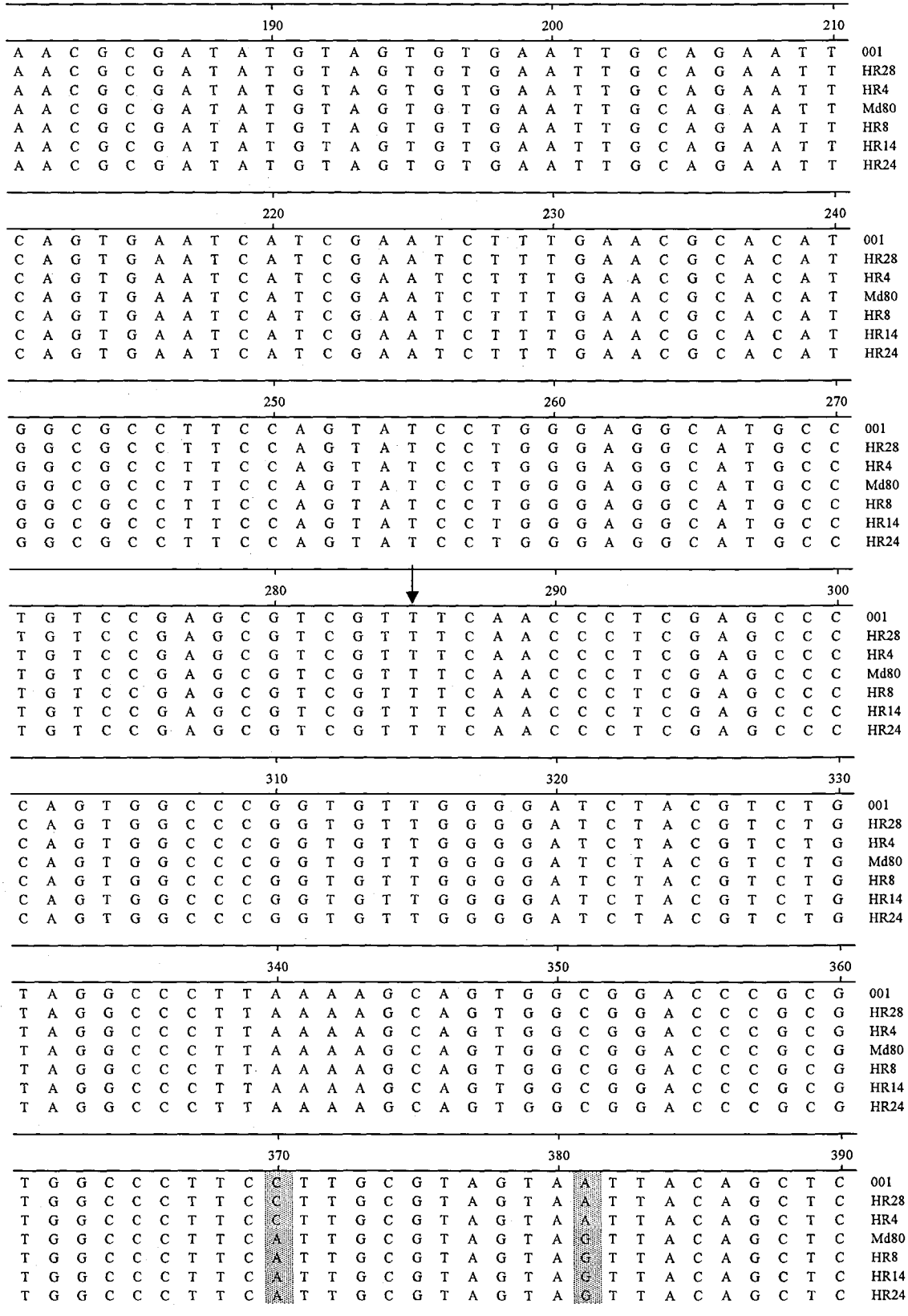

Figure 5.5. Sequence alignment of ITS1-5.8S-ITS2 of isolates of *Verticillium* isolates from horseradish (HR1, 4, 8,14, 24, 28), with previously characterised *Verticillium* isolates from horseradish (001 AFLP group β) and haploid *V. dahliae* (Md80). Shaded areas indicate sequence differences. ITS1:1-128; 5.8*S*: 129-285; ITS2: 286-452. Junctions between regions is indicated by an →. Continued.

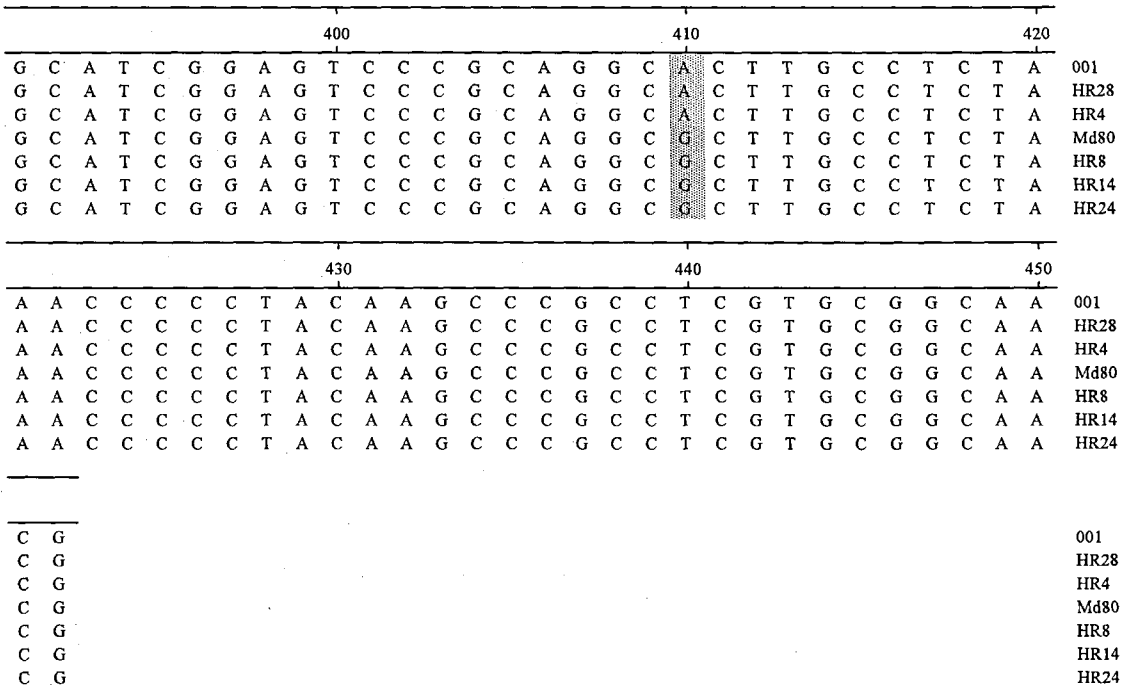

Figure 5.5. Sequence alignment of ITS1-5.8S-ITS2 of isolates of *Verticillium* isolates from horseradish (HR1, 4, 8,14, 24, 28), with previously characterised *Verticillium* isolates from horseradish (001 AFLP group β) and haploid *V. dahliae* (Md80). Shaded areas indicate sequence differences ITS1:1-128; 5.8*S*: 129-285; ITS2: 286-452. Junctions between regions is indicated by an →.

5.3.2 Identification of *Verticillium* isolates as possible 'parents'

5.3.2.1 Isolates of *V. dahliae*, *V. albo-atrum*, and *V. albo-atrum* (GpII) from Canada, and *V. tricorpus* from Israel.

DNA from these isolates was amplified using primers ITS4 and ITS5, and those which produced single products were subjected to restriction endonuclease analysis (see Table 5.2). RE analysis confirmed that the initial species designation was correct for most isolates except that of PE1855, which although it came to us as *V. albo-atrum* produced a *V. dahliae*-like restriction pattern. The amplicon for this isolate was sequenced directly, and was shown to be similar to that of sequences of *V. nigrescens*. Isolates from pea (PE6971, PE6972, PE6973) produced multiple amplicons initially; monoconidial isolates were then prepared, but multiple amplification products were again produced. This suggested that these isolates were contaminated, or at least not what they were supposed to be. This strange result has not been investigated further. Isolate Vd.LH.25 was confirmed as *V. tricorpus* by RE analysis (Table 5.2).

5.3.2.2 *V. nigrescens*, *V. theobromae* and *V. nubilum*

The ITS sequence of isolate 130213, supplied to us by the International Mycological Institute as *caveat emptor*, tested by PCR with primers ITS4 and ITS5 was identical to that for the same isolate deposited by Zare *et al.* (2000) as *V. nubilum* (GenBank Accession Nos: AJ292463). Isolates 964.95 and 8266, which came to us as *V. nubilum*, were quite distinct from 130213. Isolate 8266 bore strong similarity (98.1% identity) to deposited sequences of *V. nigrescens*, however isolate 964.95 was similar (98.6% identity) to a

deposited sequence of an unknown salal (*sic*) root associated fungus (GenBank Accession Nos. AF149073) and unlike deposited sequences for any plant pathogenic *Verticillium* species. Isolates 29778 and 225818, that came to us as *V. theobromae* were confirmed as that species (isolate 29778, 97.1% identity; isolate 225818, 86.4% identity) according to their ITS by comparison with deposited sequences on GenBank (Accession Nos. VTH292422). The species identity of isolate 15114 could not be confirmed as mixed amplicons were produced and no attempt to produce monoconidial isolates was made. PCR of rRNA IGS using primers 723 and 727 failed to produce amplicons with the isolates in this section.

5.3.3 Molecular identification of plant pathogenic *Verticillium* species

5.3.3.1 Isolates of *V. dahliae* from cauliflowers in Belgium

PCR of the ITS regions of the rRNA repeats yielded products of approximately 600 bp from all isolates tested. Sequence analysis of rRNA gene repeat ITS1-5.8*S*-ITS2 showed the isolates tested were identical with isolates from AFLP group α (Collins *et al.*, 2003) (Figure 5.6). The sequences have been deposited in GenBank (Accession Nos.: AY566600 - AY566606).

PCR of the IGS regions of the rRNA repeats gave amplicons ranging between 600 bp-800 bp. Sequence analysis of the 320 bp 'V-region' showed that the isolates tested grouped with those from AFLP group α (Collins *et al.*, 2003) (Figure 5.7). These sequences have been deposited in GenBank (Accession Nos.: AY566593 - AY566599).

5.3.3.2 Iranian *V. albo-atrum* isolates from Lucerne

Amplification by PCR using primers ITS 4 and ITS5 yielded products of approximately 600 bp from all three isolates (Ir1, Ir2, Ir3). The sequences of the complete ITS1-5.8*S*-ITS2 regions of the rRNA gene repeats for three isolates (GenBank Accession Nos.: AY536044-6) were identical to that previously reported for the Lucerne (L) pathotype of *V. albo-atrum* (Figure 5.6)

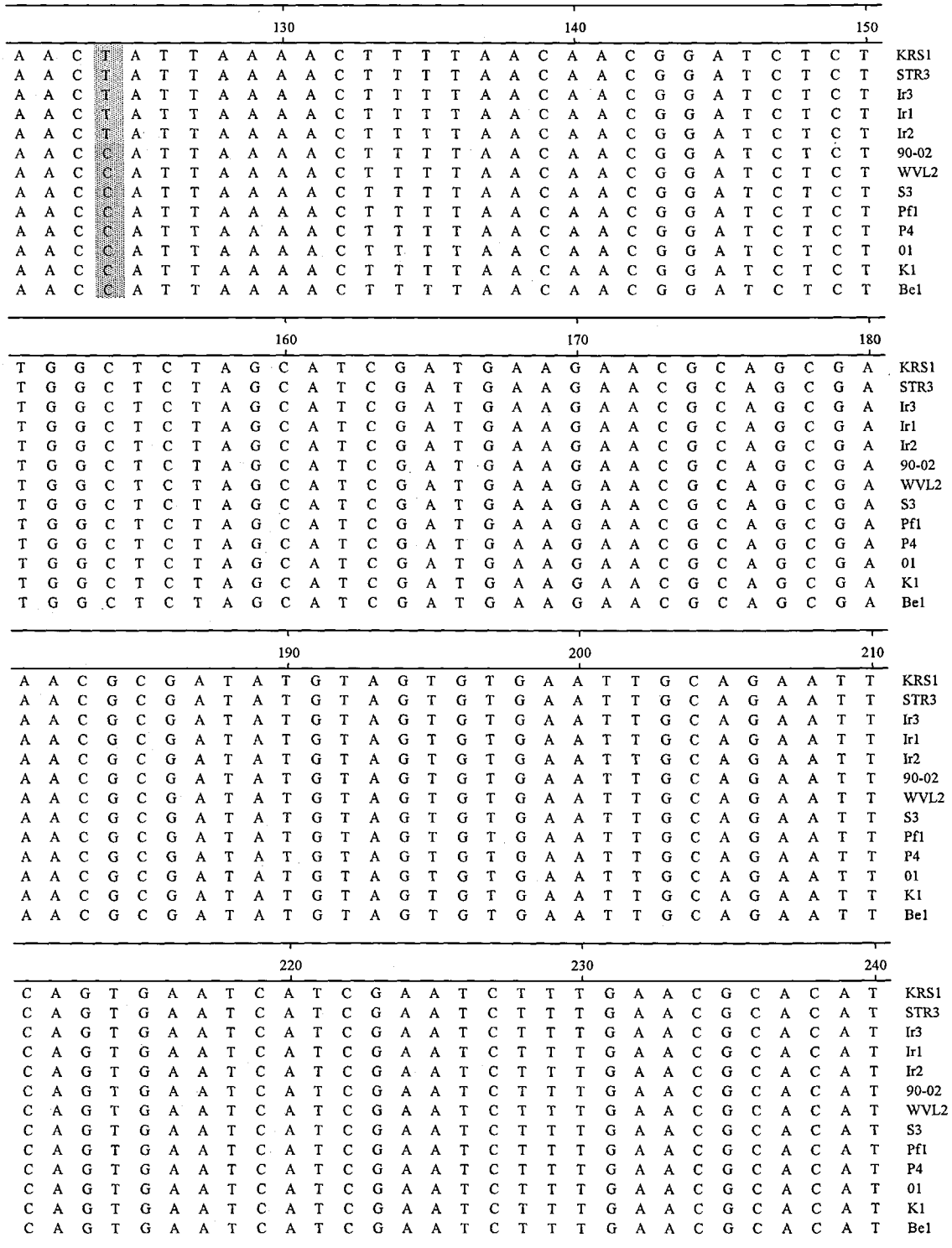

Figure 5.6. Sequence alignment of nt 120 -240 of ITS1-5.8S-ITS2 of isolates of *V. albo-atrum* from Iran (Ir1,2,3), with previously characterised *V. albo*-atrum (L) isolates STR1 and KRS1, and *Verticillium* isolates from cauliflower from Belgium (WVL2, S3, Pf1, P4, 01, K1, Be1) with a previously characterised isolate 90-02. Shaded area indicate sequence difference. No other sequence differences were seen in the complete ITS1-5.8*S*-ITS2 region

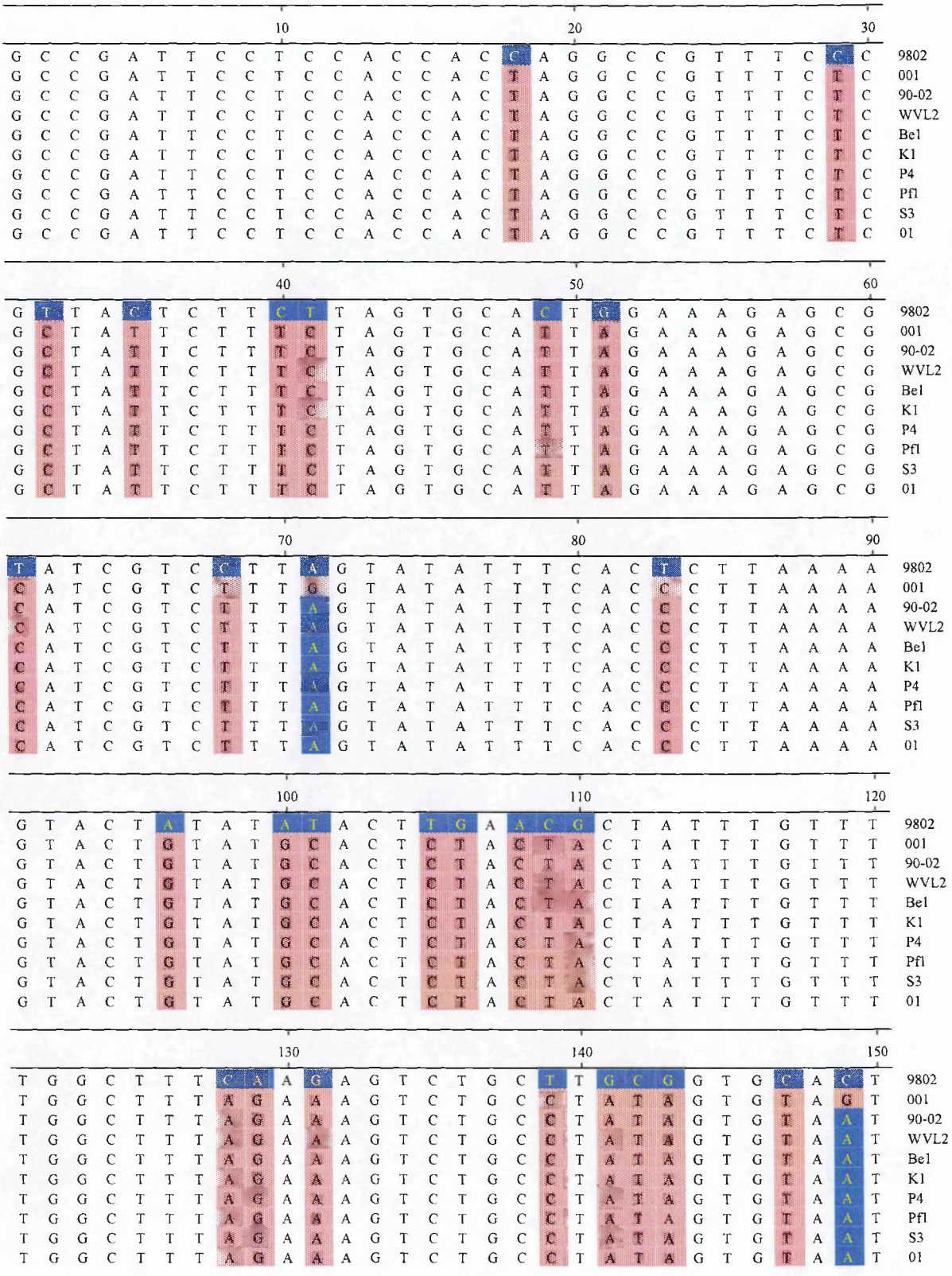

Figure 5.7. Sequence alignment of V-region of isolates of *Verticillium* isolates from cauliflower from Belgium (WVL2, S3, Pf1, P4, 01, K1, Be1) with a previously characterised isolates 90-02 (AFLP group α), 001 and 9802 (AFLP group β). Shaded areas indicate sequence differences. Continued.

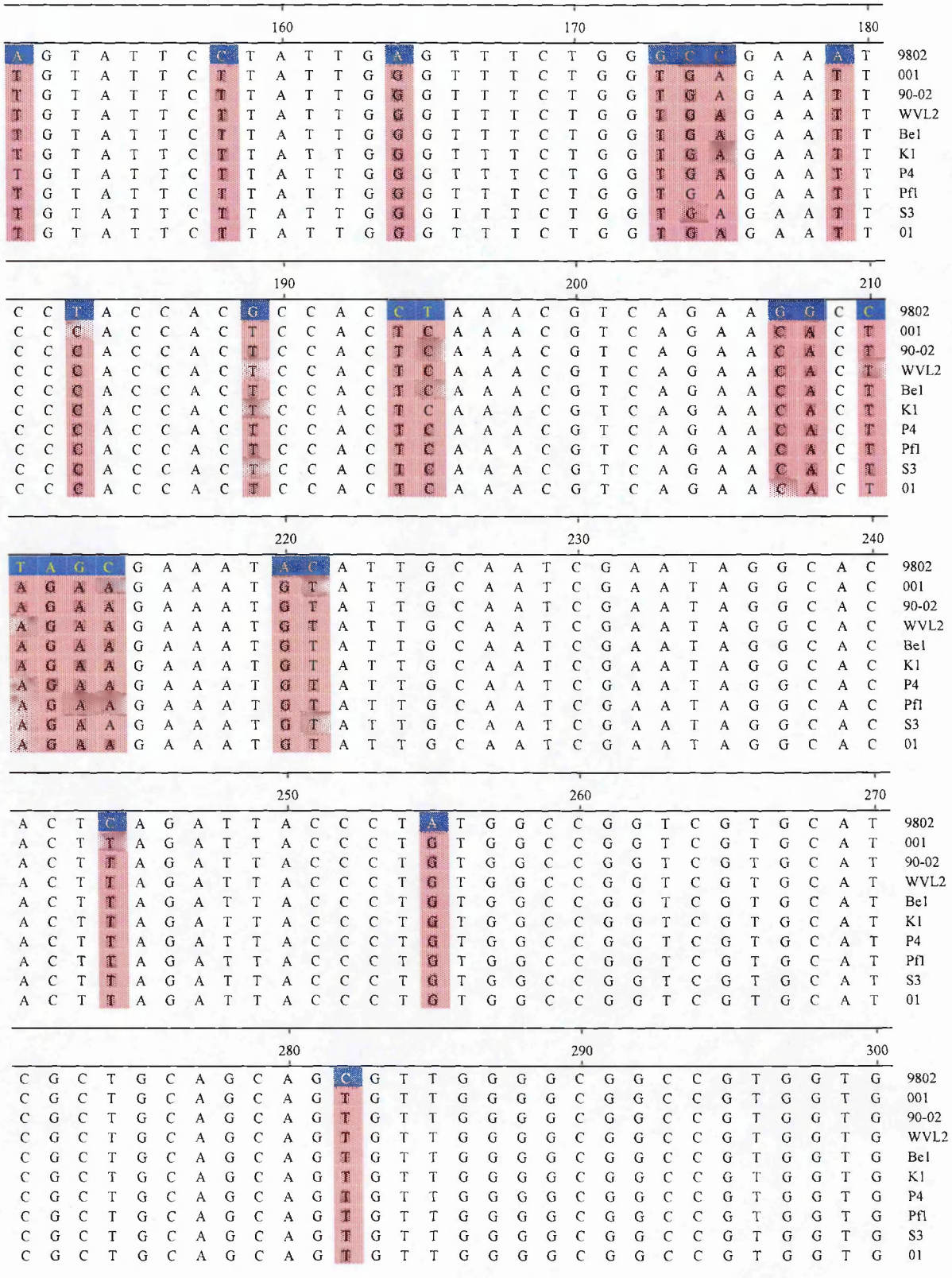

Figure 5.7. Sequence alignment of V-region of isolates of *Verticillium* isolates from cauliflower from Belgium (WVL2, S3, Pf1, P4, 01, K1, Be1) with a previously characterised isolates 90-02, 001 and 9802. Shaded areas indicate sequence differences. Continued.

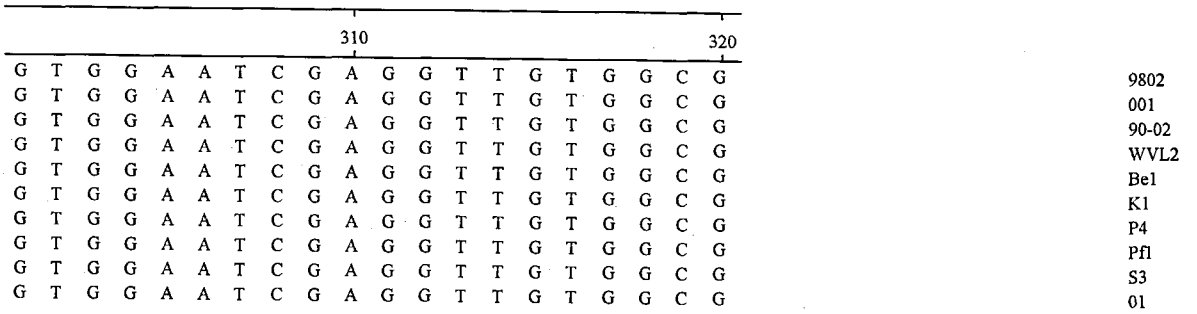

Figure 5.7. Sequence alignment of V-region of isolates of *Verticillium* isolates from cauliflower from Belgium (WVL2, S3, Pf1, P4, 01, K1, Be1) with a previously characterised isolates 90-02, 001 and 9802. Shaded areas indicate sequence differences.

5.3.4 Defoliating and Non-defoliating pathotypes

Of the amphihaploid isolates (Table 5.1 in bold) tested, only isolate 9802 produced an amplicon of 820 bp using the 'non-defoliating' primer pair. *V. albo-atrum* (L) isolates STR1 and STR3 produced an amplicons of 820 bp using the 'non-defoliating' primer pair whereas the Non-Lucerne isolates tested did not (Figure 5.8). No amplicons were produced using the 'defoliating' primer pair except from those the haploid *V. dahliae* defoliating positive controls (V-071, 1381, 1771).

Figure 5.8. Agarose gel electrophoresis of PCR products from amplification with primers 860 and 861 for the non defoliating pathotype of *V. dahliae*. Lane 1: *V. albo-atrum* (NL) isolate 1974; 2: *V. albo-atrum* (L) isolate STR3; lanes 3-5 haploid *V. dahliae* (3: Md71; 4: 12087; 5: Vd128); lanes 6 and 7: *Verticillium* amphihaploid AFLP group α isolates (6: 90-03; 7: VdII); lanes 8-11 *Verticillium* amphihaploid AFLP group β isolates (8: 001; 9: 004; 10: Md73; 11: 9802) Pc : Primer control; M: Molecular marker.

5.4 Discussion

Where relevant to this chapter, previous studies (e.g. Karapapa *et al.*, 1997; Collins *et al.*, 2003) have tended to focus on the two main plant pathogens, *V. dahliae* and *V. albo-atrum* as possible 'parents' of the hybrids. The extended studies of related minor species has not only revealed the unexpected variability in some of the 'species' but also ruled out their being involved in hybridisation events thought to have given rise to the amphihaploids, suggesting a previously unrecognised species may exist (see Barbara and Clewes (2003) for further discussion of this).

As indicators of 'interesting' the non-coding regions of the rRNA gene repeats provide a solid basis from which future studies can be based, by rapidly identifying possible 'parents' or 'new' amphihaploid isolates. Studies of the ITS and V-regions did not suggest that any of the isolates tested here were of further interest and there appeared to be no new groups of crucifer isolates or potential *V. albo-atrum*-like 'parent(s)' in this group.

However, this does pose some interesting questions as to the nature of the parental isolates. Limited molecular evidence and past assumptions (see General Introduction) had previously linked *V. albo-atrum* and *V. dahliae* in the hybridisation and generation of amphihaploid isolates. However the more recent molecular data (e.g. V-region) clearly differentiate the non-*V. dahliae* parent from *V. albo-atrum*. But why are isolates molecularly like this hypothetical parent not being isolated from the field? Maybe the answer can be found in the weeds, or soil saprophytes that are not identified as they cause no significant problems or this 'parent' may occur in some geographically little studied (as far as wilt disease is concerned) region. However should an intensive survey be conducted of isolates from weed, new hosts or other regions the non-coding regions of rRNA genes

should be considered generally reliable and useful starting points from which all molecular studies should be based.

As an initial observation the studies in this chapter tell us that *caveat emptor* should always be taken into account with data from GenBank, cultures brought from culture collections, and those supplied by other research groups. Too often it is plain to see in literature this is not the case when authors interpret results based on the initial species designation which may be correct but is more likely not to be correct (*e.g.* Bidochka *et al.*, 1999). Similarly where reported properties of isolates clash with those expected it is useful to establish the molecular identity of the isolates concerned. Here, for example the two Polish isolates supplied as *nit* mutants were said to come from oilseed rape. However, long-spored isolates have proved too refractory for the production of mutants (Clarkson and Heale, 1985a,b; Hastie, 1973; Ingram 1968; Nagao *et al.*, 1994; Puhalla and Hummel, 1983; Subbarao *et al.*, 1995) so it was no surprise that these two are molecularly indistinguishable from the majority of 'ordinary' haploid *V. dahliae*. Whether these isolates did originally come from oilseed rape could not be tested. The other two Polish isolates, from which no *nit* mutants had been derived appear to be indistinguishable, by rRNA genes at least, from other AFLP group α amphihaploid isolates from oilseed rape.

The isolates from horseradish in Illinois were particularly interesting. Short-spored isolates purportedly coming from crucifers have been supplied to Warwick HRI in the past but in general these have come long after initial isolation and via third parties. There has therefore been some doubt about whether short-spored crucifer isolates do exist. At the 8th International *Verticillium* Symposium held in Cordoba, Spain, Dr. R. Bhat reported isolates of *V. dahliae* from horseradish that could be placed into VCG 2B. Those supplied to

Warwick HRI by Dr W. Chen, proved to be a mixture of isolates molecularly like the horseradish isolates from Illinois (isolates 001 and 004) that were already available at Warwick HRI and others (presumably short-spored ones although this was not checked) like haploid *V. dahliae*. This is the best example we have of the existence of both short-spored and long-spored isolates naturally infecting one cruciferous host

One group of isolates has not been entirely ruled out as of interest to the main studies of this thesis. The pea isolates from Canada were included as they were quite divergent in the studies of Mahuku and Platt (2002). In this work they produced multiple amplicons, even when monoconidial isolates were used. Time constraint hindered further investigation of this strange result.

The great molecular diversity among the isolates supposedly *V. nubilum* raises interesting questions as to which is the 'true' species. These questions are not directly related to this thesis but have been more fully discussed in a review inspired by thesis results presented here and in Collins (2002) (Barbara and Clewes, 2003) .

Also, although not directly relevant to the main thrust of this thesis the results showing the Belgian cauliflower isolates are not readily distinguished from European oilseed rape isolates and suggest this new disease (for Europe) has arisen as a result of oilseed rape isolates infecting cauliflower, even though oilseed rape has not been grown near the sites of the infected cauliflowers (Prof. Dr. M. Höfte and Dr. J. Debode personal communication). Californian cauliflower isolates are very similar (Collins *et al.*, 2003) and an alternative possibility is that the disease was imported from the US in cauliflower (on

seed?) although no evidence of a direct link of this type was available. This result has been published as part of a study on this new disease in Belgium (Debode *et al.*, 2004).

The initial communication received from Iran suggested that the recent isolates from Lucerne in that country were unlike other *V. albo-atrum* (L) isolates and had been tentatively ascribed to a new species (Dr. M. Ghalander personal communication). This makes them of potential interest as the non–*V. dahliae* parent, and of intrinsic taxonomic interest. The results presented here clearly show the three isolates studied to be molecularly indistinguishable from other *V. albo-atrum* (L) isolates and not a new species. Whilst reducing their intrinsic interest, it raises questions as to how these host-specific isolates (based on the characteristics of the other L pathotypes isolated) reached Iran where the disease was previously unknown. These results have been published as part of a new record of this disease (Ghalander *et al.*, 2004).

5.5 Concluding remarks

- Isolates of *Verticillium* from cauliflower in Belgium are molecularly similar to those from oilseed rape and cauliflower in California.
- Isolates of *V. albo-atrum* from Lucerne in Iran are molecularly similar to other *V. albo-atrum* (L) isolates.
- *V. nubilum*, *V. nigrescens* and *V. theobromae* are intraspecifically divergent but no studies to further refine the taxonomy of these isolates were undertaken.
- It seems that that there are no 'new' amphihaploid *Verticillium* isolates that have arisen independently of those from AFLP groups α and β.
- Isolates like the putative non-*V. dahliae*-like 'parents' of the amphihaploid isolated are not being isolated from the field.

6 MOLECULAR EVIDENCE FOR THE HYBRID NATURE AND PARENTAL ORIGINS OF *VERTICILLIUM* AMPHIHAPLOID ISOLATES

6.1 Introduction

Verticillium amphihaploids and their related species have been studied molecularly using many different molecular markers (Morton *et al.*, 1995a,b; Karapapa *et al.*, 1997; Karapapa and Typas, 2001; Zeise and Von Tiedemann, 2002a; Collins *et al.*, 2003; Fahleson *et al.*, 2004) but there has been no published direct molecular evidence that these isolates are interspecific hybrids, and it has been a key aim of these studies to find a marker that is retained from both parents in the hybrid. In such a situation where both 'parents' of the hybrid were known this could be used to identify a hybrid or conversely used to ascertain a possible parent. Collins *et al.* (2003) looked for the presence of 'minor' ITS types within functional rRNA gene tandem arrays. Some isolates of *V. dahliae* and *Verticillium* amphihaploids did seem to retain a minor-type rRNA *V. albo-atrum*-like component, however its amplification was variable and it was not clear whether it could be discounted as contamination or whether weak amplification was due to competition with the predominant ITS type in the PCR. Only a single isolate from AFLP group β, Md73, consistently gave amplification every time that it was tested.

6.1.1 Concerted evolution

Concerted evolution describes the homogenisation of a multigene family that can occur at several levels, depending on the organisation of the repeats. The simplest form of homogenisation is within a tandemly repeated array and is known as intralocus

homogenisation, an example of which is the ITS region of the functional rRNA gene repeat unit. In many organisms multigene families are organised into two or more arrays within the genome, and homogenisation can occur between these arrays - interlocus homogenisation. Evidence of interlocus homogenisation is found in the functional rRNA gene arrays of hybrids, such as the majority of *Verticillium* amphihaploid isolates (Collins *et al.*, 2003) where dissimilar rRNA arrays are brought together in one hybrid genome and one is 'selected for'(Hillis *et al.*, 1991; Wendel *et al.*, 1995).

6.1.2 5*S* rRNA gene

The 5*S* rRNA gene encodes an RNA transcript of *ca.* 120 nt. These genes are also repeated, but the structure of their organisation is more diverse than those of the major (large-5.8*S*-small) subunit rRNA gene repeat. In some fungi, the 5*S* rRNA gene is located within the major rRNA gene repeat unit but in others is found as dispersed individual copies. In eukaryotes, the major rRNA gene repeat are transcribed separately from the 5*S* rRNA gene by a different polymerase, RNA polymerase I as opposed to RNA polymerase III. Given that the 5*S* rRNA gene is transcribed by a different RNA polymerase, it is hardly surprising that in many eukaryotic species it is not linked to the functional rRNA gene repeat unit. In the plant pathogenic *Verticillium,* the 5*S* rRNA gene is not located in the major rRNA gene repeat unit (Mukhamedov *et al.*, 1990).

Epichloë typhina isolate E8 and *Neotyphodium lolii* isolate Lp5 were found to be the natural parents of the hybrid *Epichloë* endophyte, Lp1. In this hybrid isolate the 5*S* rRNA genes were found not to be in tandem array but were dispersed throughout the genome (Ganley and Scott, 2002). However, in this scenario both 'parents' of this isolate were

known and it was established that only one parental 5*S* rRNA gene type was found in the hybrid, although it was found that there were two sub-groups of the gene indicating a degree of microheterogeneity as found in *Aspergillius nidulans* (Bartoszewski *et al.*, 1987); this is an example of interlocus homogenisation.

In the nuclear genome of higher plants, the 5*S* rRNA genes are present in multiple copies arranged in tandem arrays (Ganal *et al.*, 1988). *Nicotiana tabacum* is an allotetraploid derived from the ancestors of the modern diploids *N. sylvestris* and *N. tomentosiformis*. Studies by Fulneček *et al.* (2002) characterised two distinct families of 5*S* rRNA genes in *N. tabacum*; one family had an average 431 bp unit length and the other 646 bp unit length. The diploid species, *N. sylvestris* and *N. tomentosiformis*, were found to have unit lengths of 431 bp and 644 bp respectively. The non-coding region of the short sequence in *N. tabacum* showed high sequence similarity to *N. sylvestris* (S genome sub-region) and the longer sequence shared high similarity with *N. tomentosiformis* (T genome sub-region). The authors suggest that this indicates that the two 5*S* rRNA gene families have their origin in diploid ancestor species. Sequence analysis found that there was as much diversity within the 5*S* rRNA gene family units within the diploid species as there was within the T and S-genome 5*S* rRNA gene units, suggesting 5*S* rRNA gene diversification before *N. tabacum* speciation. The authors conclude that there is no evidence of interlocus homogenisation of the two 5*S* rRNA gene families in *N. tabacum*.

6.1.3 β-tubulin gene

A number of fungal β-tubulin genes have been isolated and found to encode highly homologous proteins. In a few species conservation of gene structure has been

demonstrated, with introns located in identical positions in the β-tubulin genes of different fungal species (Orbach *et al.*, 1986; May *et al.*, 1987; Smith *et al.*, 1988, Byrd *et al.*, 1990, Cooley and Caten, 1993; Nowak and Kuck, 1994). Most fungal species only contain a single β-tubulin gene, there are however two genes found in *Aspergillus nidulans, Trichoderma viride, Cryptococcus neoformans, Colletotrichum graminicola, Colletotrichum gloeosporoides* f. sp. *aeschynome* and *Galactomyces geotrichum* (May *et al.*, 1987; Pannaccione and Hanau, 1990; Gold *et al.*, 1991; Goldman *et al.*, 1993; Buhr and Dickman, 1993, 1994; Cruz and Edlind, 1997). Within these species, of the two genes found, one is similar to β-tubulin genes found in related species whilst the other is more divergent.

The two distinct β-tubulin genes in *A. nidulans* have been shown to be functionally interchangeable (May, 1989) but the more atypical gene of the two is expressed at a lower level (Panccione *et al.*, 1990; Cruz and Edlind, 1997) and shows a restricted expression pattern *e.g.* expressed during sporulation but not vegetative growth (May *et al.*, 1987; Buhr and Dickman, 1994).

Analysis of the β-tubulin gene has demonstrated its phylogenetic utility at the interspecific level in fungi (Shardl *et al.*, 1994; Tsai *et al.*, 1994; O'Donnell and Cigelnik, 1997; Ayliffe *et al.*, 2001; Kim *et al.*, 2003; Corradi *et al.*, 2004). These genes are suited to phylogenetic analysis as the high conservation of the β-tubulin proteins should make alignments simple with few deletions and insertions occurring (Edlind *et al.*, 1996; Thon and Royse, 1999). However, introns within β-tubulin gene were found to be twice as informative as exons (O'Donnell and Cigelnik, 1997).

6.1.4 Mitochondrial cytochrome B gene

Mitochondrial (mt) genes are an attractive marker for inferring similarities between closely related species due to the rapid evolution of the mitochondrial genome, the lack of recombination and, in sexual organisms, the strict maternal inheritance (Manceau *et al.*, 1999). RFLP analysis of mtDNA has been shown to be useful in determining the relationships between fungi. Collins *et al.* (2003) established mtDNA RFLP patterns for *Verticillium* amphihaploid isolates, *V. dahliae* and *V. albo-atrum. V. albo-atrum* Lucerne isolates were clearly differentiated from the Non-Lucerne isolates and both of these pathotypes were differentiated from haploid *V. dahliae*. The majority of AFLP group α amphihaploid isolates, and two (Md73 and 9802) from AFLP group β clustered together and were more similar to *V. albo-atrum* than *V. dahliae*. However, isolates of AFLP group β from Illinois horseradish (001 and 004) were more similar to haploid *V. dahliae*.

Very limited studies, undertaken by Dixelius' group and published recently by Fahleson *et al.* (2004), of amphihaploid isolates taken from mainly oilseed rape in Northern Europe and cauliflower isolates from California (which one could assume to all be AFLP group α) alongside isolates of *V. dahliae*, *V. albo-atrum*, *V. tricorpus,* and *V. nigrescens* showed that at this marker there was similarity between the amphihaploid isolates from Northern Europe and haploid *V. dahliae* and *V. albo-atrum*. However, the authors' state that *V. albo-atrum* is closely related to the amphihaploid isolates and disregard the apparent similarity of *V. dahliae* to the long-spored *Verticillium* isolates.

6.1.4 Aims and Objectives

- To develop molecular markers that are retained in the amphihaploid isolates from both 'putative' parent species.
- Use the molecular markers to understand the relationship of the amphihaploid isolates to related species.
- Produce a molecular 'characterisation' of the putative 'parent' species to enable its future identification.

6.2 Methods

6.2.1 PCR amplification

The majority of the coding sequence of the 5*S* rRNA gene was amplified by primers 958 and 959. Primers 846 and 847 are positioned within the 5*S* rRNA gene coding region but 'facing outwards' and were used to amplify away from the gene (Figure 6.1a) (Table 2.1).

Primers for the β-tubulin gene as described by O'Donnell and Cigelnik (1997) were tested in all combinations (Figure 6.1b). Primers 945 (bTub11) and 946 (bTubT2) (Table 2.1) amplified a partial sequence of the β-tubulin gene and produced an apparently interesting result. All subsequent analyses used this primer pair.

Primers 955 and 957 amplified the majority of the mitochondrial cytochrome B gene and were designed from an alignment of mitochondrial cytochrome B gene sequences of related genera published on the NCBI GenBank database (Accession numbers: AY285743 (*Glomerella graminicola*), AY245425 (*Magnaporthe grisea*), AB025529 (*Paecilomyces farinosus*), AB020004 (*Aspergillus niger*), AB025494 (*Emericella purpurea*), AB025479 (*Eurotium tonophilum*), AB025526 (*Neosartorya fischeri*), AB025901 (*Cladosporium carrionii*), AB025890 (*Exophiala moniliae*)). (Table 2.1).

DNA extractions and PCR conditions were as described (see 2.3 and 2.4). Annealing temperatures were as follows primers 846 and 847 (5*S* rRNA IGR) 57°C, primers 945 and 946 (β-tubulin gene) 58°C, primers 955 and 957 (mitochondrial cytochrome B gene) 46°C). PCR products from amplification with 846/847, and 945/946 were analysed by

electrophoresis on a 2.5% agarose (w/v) gel. PCR products were purified using the QIAquick PCR Purification Kit (Qiagen Ltd, UK). The concentrations of purified PCR-amplified DNAs were estimated following electrophoresis by comparison of fluorescence to that of known quantities of a low DNA mass ladder (Invitrogen, UK).

6.2.2 Sequence analysis

Purified PCR products were sequenced directly in one direction except where initial sequences indicated the PCR products to be mixed; these were cloned for resequencing and further analysis. Purified PCR products of the 846/847 were cloned into pCR®4-TOPO® using the TOPO-TA Cloning® Kit for Sequencing (Invitrogen). Purified PCR products of the 5*S* rRNA gene and parts of the β-tubulin gene were cloned into pSTBlue-1 using the AccepTor™ Vector Kit (Novagen). To verify the insert DNA, transformants were picked and transferred to a replica LB plate with required antibiotics. Following transfer the cocktail sticks used were agitated in 60 μl RO water, the stick removed and the water boiled for 5 min, centrifuged for 1 min at maximum speed and 5μl was used in subsequent PCR amplifications. Where the presence of the insert had been confirmed by PCR, colonies were picked from the replica plate using a flamed loop and inoculated into 2ml LB broth with antibiotics (as described in the manufacturer's instructions) in a sterile falcon tube. The tubes were incubated overnight in an orbital shaker maintained at 37°C at 200 rpm. Plasmid DNA was extracted using Qiagen miniprep kit (Qiagen Ltd, UK).

Inserts in plasmid DNA were sequenced directly in both directions using universal primers and the BigDye® v3.1 terminator chemistry (Applied Biosystems, USA) according to the manufacturers' instructions and ABI PRISM®3100 Genetic Analyzer (Applied

Biosystems, USA). Sequences were edited and aligned using Lasergene v5.07 (DNAStar Inc. USA).

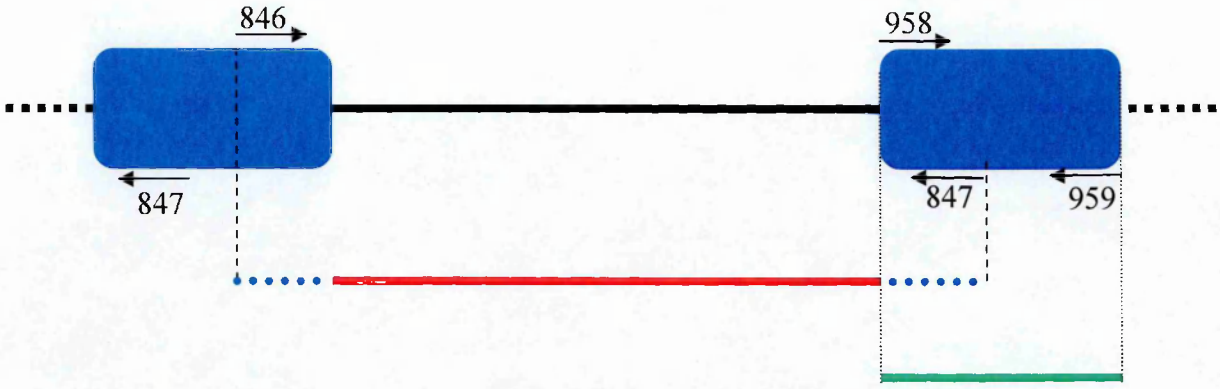

Figure 6.1a. Schematic diagram representing two 5*S* rRNA genes separated by an intergenic region with the relative positions of the primers. Also shown are the expected PCR products with regard to the primer combinations used. ▬ Is the expected product from amplification of primers 958/959 for the 5*S* rRNA gene whereas ▬ is the expected amplicon from amplification with 846/847 for the 5*S* rRNA IGR. ••• shows that with 846/847 parts of the 5*S* rRNA gene are also amplified.

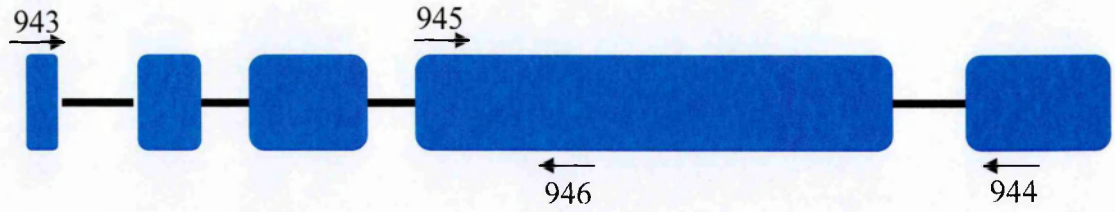

Figure 6.1b. Map of the β-tubulin gene of *Fusarium* sp. from O'Donnell and Cigelnik (1997) with relative positions of the primers tested. ■ Indicates exons, ▬ indicates introns.

Clone	Isolate	AFLP group	Gene
42	12087	Haploid *V.dahliae*	5S rRNA IGR
43	12087	Haploid *V.dahliae*	5S rRNA IGR
46	12087	Haploid *V.dahliae*	5S rRNA IGR
47	12087	Haploid *V.dahliae*	5S rRNA IGR
48	12087	Haploid *V.dahliae*	5S rRNA IGR
50	12087	Haploid *V.dahliae*	5S rRNA IGR
51	12087	Haploid *V.dahliae*	5S rRNA IGR
72	Vd128	Haploid *V.dahliae*	5S rRNA IGR
74	Vd128	Haploid *V.dahliae*	5S rRNA IGR
75	Vd128	Haploid *V.dahliae*	5S rRNA IGR
77	Vd128	Haploid *V.dahliae*	5S rRNA IGR
78	Vd128	Haploid *V.dahliae*	5S rRNA IGR
79	Vd128	Haploid *V.dahliae*	5S rRNA IGR
84	Vd128	Haploid *V.dahliae*	5S rRNA IGR
86	Vd128	Haploid *V.dahliae*	5S rRNA IGR
92	VdII	Amphihaploid α	5S rRNA IGR
93	VdII	Amphihaploid α	5S rRNA IGR
94	VdII	Amphihaploid α	5S rRNA IGR
95	VdII	Amphihaploid α	5S rRNA IGR
96	VdII	Amphihaploid α	5S rRNA IGR
97	VdII	Amphihaploid α	5S rRNA IGR
98	VdII	Amphihaploid α	5S rRNA IGR
99	VdII	Amphihaploid α	5S rRNA IGR
121	001	Amphihaploid β	5S rRNA IGR
122	001	Amphihaploid β	5S rRNA IGR
123	001	Amphihaploid β	5S rRNA IGR
124	001	Amphihaploid β	5S rRNA IGR
126	001	Amphihaploid β	5S rRNA IGR
133	001	Amphihaploid β	5S rRNA IGR
140	9802	Amphihaploid β	5S rRNA IGR
141	9802	Amphihaploid β	5S rRNA IGR
143	9802	Amphihaploid β	5S rRNA IGR
145	9802	Amphihaploid β	5S rRNA IGR
146	9802	Amphihaploid β	5S rRNA IGR
147	9802	Amphihaploid β	5S rRNA IGR
148	9802	Amphihaploid β	5S rRNA IGR
149	9802	Amphihaploid β	5S rRNA IGR
155	9010	Amphihaploid α	5S rRNA IGR
156	9010	Amphihaploid α	5S rRNA IGR
157	9010	Amphihaploid α	5S rRNA IGR
158	9010	Amphihaploid α	5S rRNA IGR
159	9010	Amphihaploid α	5S rRNA IGR
160	Md71	Haploid *V.dahliae*	5S rRNA IGR
161	Md71	Haploid *V.dahliae*	5S rRNA IGR
162	Md71	Haploid *V.dahliae*	5S rRNA IGR
163	Md71	Haploid *V.dahliae*	5S rRNA IGR
164	Md71	Haploid *V.dahliae*	5S rRNA IGR
167	P14	Haploid *V.dahliae*	5S rRNA IGR
169	P14	Haploid *V.dahliae*	5S rRNA IGR
171	P14	Haploid *V.dahliae*	5S rRNA IGR
172	P14	Haploid *V.dahliae*	5S rRNA IGR
173	P14	Haploid *V.dahliae*	5S rRNA IGR
175	P14	Haploid *V.dahliae*	5S rRNA IGR
176	004	Amphihaploid β	5S rRNA IGR
177	004	Amphihaploid β	5S rRNA IGR
178	004	Amphihaploid β	5S rRNA IGR

180	004	Amphihaploid β	5S rRNA IGR
181	004	Amphihaploid β	5S rRNA IGR
182	004	Amphihaploid β	5S rRNA IGR
183	004	Amphihaploid β	5S rRNA IGR
185	004	Amphihaploid β	5S rRNA IGR
187	Md73	Amphihaploid β	5S rRNA IGR
188	Md73	Amphihaploid β	5S rRNA IGR
189	Md73	Amphihaploid β	5S rRNA IGR
190	Md73	Amphihaploid β	5S rRNA IGR
191	Md73	Amphihaploid β	5S rRNA IGR
193	Md73	Amphihaploid β	5S rRNA IGR
194	Md73	Amphihaploid β	5S rRNA IGR
195	Md73	Amphihaploid β	5S rRNA IGR
312	84020	Amphihaploid α	5S rRNA IGR
313	84020	Amphihaploid α	5S rRNA IGR
314	84020	Amphihaploid α	5S rRNA IGR
316	84020	Amphihaploid α	5S rRNA IGR
317	84020	Amphihaploid α	5S rRNA IGR
318	84020	Amphihaploid α	5S rRNA IGR
320	84020	Amphihaploid α	5S rRNA IGR
321	84020	Amphihaploid α	5S rRNA IGR
541	Md80	Haploid *V.dahliae*	5S rRNA IGR
542	Md80	Haploid *V.dahliae*	5S rRNA IGR
544	Md80	Haploid *V.dahliae*	5S rRNA IGR
546	Md80	Haploid *V.dahliae*	5S rRNA IGR
547	Md80	Haploid *V.dahliae*	5S rRNA IGR
548	Md80	Haploid *V.dahliae*	5S rRNA IGR
549	Md80	Haploid *V.dahliae*	5S rRNA IGR
500	Md71	Haploid *V.dahliae*	β-tubulin
501	Md71	Haploid *V.dahliae*	β-tubulin
503	Md71	Haploid *V.dahliae*	β-tubulin
504	Md71	Haploid *V.dahliae*	β-tubulin
279	Md71	Haploid *V.dahliae*	β-tubulin
506	12087	Haploid *V.dahliae*	β-tubulin
507	12087	Haploid *V.dahliae*	β-tubulin
508	12087	Haploid *V.dahliae*	β-tubulin
509	12087	Haploid *V.dahliae*	β-tubulin
510	STR1	*V. albo-atrum*	β-tubulin
511	STR1	*V. albo-atrum*	β-tubulin
512	STR1	*V. albo-atrum*	β-tubulin
513	STR1	*V. albo-atrum*	β-tubulin
514	STR1	*V. albo-atrum*	β-tubulin
515	Vd128	Haploid *V.dahliae*	β-tubulin
517	Vd128	Haploid *V.dahliae*	β-tubulin
518	Vd128	Haploid *V.dahliae*	β-tubulin
520	VA1	*V. albo-atrum*	β-tubulin
521	VA1	*V. albo-atrum*	β-tubulin
523	VA1	*V. albo-atrum*	β-tubulin
532	P14	Haploid *V.dahliae*	β-tubulin
533	P14	Haploid *V.dahliae*	β-tubulin
534	P14	Haploid *V.dahliae*	β-tubulin
537	Md80	Haploid *V.dahliae*	β-tubulin
538	Md80	Haploid *V.dahliae*	β-tubulin
539	Md80	Haploid *V.dahliae*	β-tubulin
222	VdII	Amphihaploid α	β-tubulin
223	VdII	Amphihaploid α	β-tubulin
224	VdII	Amphihaploid α	β-tubulin
226	VdII	Amphihaploid α	β-tubulin
227	VdII	Amphihaploid α	β-tubulin

253	9010	Amphihaploid α	β-tubulin
255	9010	Amphihaploid α	β-tubulin
256	9010	Amphihaploid α	β-tubulin
257	9010	Amphihaploid α	β-tubulin
258	9010	Amphihaploid α	β-tubulin
245	84020	Amphihaploid α	β-tubulin
247	84020	Amphihaploid α	β-tubulin
249	84020	Amphihaploid α	β-tubulin
250	84020	Amphihaploid α	β-tubulin
251	84020	Amphihaploid α	β-tubulin
202	9802	Amphihaploid β	β-tubulin
205	9802	Amphihaploid β	β-tubulin
207	9802	Amphihaploid β	β-tubulin
211	9802	Amphihaploid β	β-tubulin
212	001	Amphihaploid β	β-tubulin
213	001	Amphihaploid β	β-tubulin
214	001	Amphihaploid β	β-tubulin
215	001	Amphihaploid β	β-tubulin
216	001	Amphihaploid β	β-tubulin
217	001	Amphihaploid β	β-tubulin
219	001	Amphihaploid β	β-tubulin
221	001	Amphihaploid β	β-tubulin
261	Md73	Amphihaploid β	β-tubulin
264	Md73	Amphihaploid β	β-tubulin
265	Md73	Amphihaploid β	β-tubulin
270	Md73	Amphihaploid β	β-tubulin

Table 6.1. Identities of clones sequenced in studies of 5*S* rRNA and β-tubulin genes. Full details of isolates are given in Appendix 9.2. AFLP groups of amphihaploids are as given in Collins *et al.* (2003).

6.2.3 Southern analysis of 5S rRNA gene and β-tubulin gene

6.2.3.1 Endonuclease restriction digest of genomic DNA

The restriction enzymes *Pvu*I and *Nru*I were chosen for this study as both were found not to cut within the β-tubulin sequence amplified but *Pvu*I was found to cut within the larger amphihaploid sequence of the 5*S* rRNA IGR, and *Nru*I to cut within the *V. dahliae*-derived short sequence of the 5*S* rRNA IGR. The digest comprised of 5 μg genomic DNA, 30U enzyme (*Pvu*I or *Nru*I), 1 x REact®7 buffer, 10mg/ml spermidine and RO water to a total volume of 200 μl. The reaction was left overnight at 37°C. The digestion components were mixed with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1), centrifuged at maximum speed for 2 minutes, and the aqueous phase removed. To this, an equal volume of chloroform was added, mixed and centrifuged for 2 minutes at maximum speed. The aqueous phase was removed and mixed with an equal volume of chloroform and centrifuged at maximum speed. The aqueous phase was removed and mixed with two volumes of 100% ethanol, 0.1M NaCl in a 0.5 ml microcentrifuge tube. Tubes were stored at -20°C overnight. Tubes were centrifuged at maximum speed for 10 minutes, and the pellets washed twice in cold 70% ethanol. DNA pellets were air dried briefly and resuspended in 30 μl RO water. DNA concentrations were measured using the NanoDrop ND-1000 spectrophotometer (NYXORbiotech, France).

6.2.3.2 Southern blotting

Two 1% (w/v) agarose gels prepared with 1 x TBE (without ethidium bromide) were loaded with the digestion products. The first gel was loaded with genomic DNA for each isolate digested with *Pvu*I and the second with genomic DNA for each isolate digested

with *Nru*I. For each isolate approximately 2 μg DNA was loaded for each enzyme. Gels were run for 900Vhr, stained in 1 x TBE, 0.33 μg/µl ethidium bromide for 1h, photographed and then destained in RO water. The blotting was performed by alkaline transfer onto Hybond N+ (Amersham) according to the manufacturer's instructions.

6.2.3.3 Hybridisation between N+ filters and α^{32}P dUTP labelled DNA probes

The Hybond N+ membranes were placed onto nylon mesh that had been wetted in 2 x SSC (saline sodium citrate) rolled and placed into hybridisation tubes in a rotisserie oven. 50 ml of hybridisation buffer (0.5M Na phosphate pH 7.2, 70 g/l sodium dodecyl sulphate (SDS); 10mM EDTA, pH 8.0 and 100 μg/ml single stranded salmon sperm DNA) was added to the tubes at 65°C and left to prehybridise for 3h. RNA probes were produced for β-tubulin and 5*S* rRNA gene sequence and simultaneously labelled with α ^{32}P dUTP using the Strip-EZ™ RNA Kit (Ambion (UK) Ltd, UK). The labelled RNA probes were separated from the other reaction components by a Sephadex G50 column and counted using a scintillation counter. The 50 ml hybridisation buffer was replaced with 10ml fresh hybridisation buffer and 2 x 10^7 counts per minute labelled probe added and left overnight at 65°C. The blots were washed with 2 x SSC and 1% SDS three times at 65°C. The filters were wrapped in Saran Wrap (Dow Chemicals) and placed into X-ray cassettes with intensifying screens and with XAR5 film (Kodak) under a safelight. The cassettes were placed at -80°C, and the film was developed after varying periods. Membranes were stripped of the RNA probes according to the Strip-EZ™ RNA Kit protocol (Ambion (UK) Ltd, UK).

6.2.3.4 Analysis of 5*S* rRNA gene Southern blot profiles

The DNA profiles generated from the different enzymes probed with 5*S* rRNA gene were scored manually as follows: 1 if a specific molecular weight band was present and 0 if it was absent. The data was analysed using Genstat (Version 6, Lawes Agricultural Trust, Rothamsted, UK) using an UPGMA approach and Jaccard's coefficient. Results from cluster analysis are presented as a dendrogram to display genetic distance between isolates.

6.3 Results

6.3.1 Mitochondrial cytochrome B gene

Amplification of the mitochondrial cytochrome B gene with primers 955 and 957 produced single amplicons with all isolates tested (Figure 6.2). Sequence analysis of the amplicons showed that *V. albo-atrum* isolates were distinct from *V. dahliae* and *Verticillium* crucifer isolates. Sequence analysis also split isolates from AFLP group β into two (Figure 6.3a). Isolates from AFLP group α clustered together with the majority of haploid isolates from *V. dahliae*. Isolates of *V. nubilum*, *V. nigrescens* and *V. theobromae* were only distantly related at this locus (data not shown for *V. nigrescens* and *V. theobromae*).

Figure 6.2. Agarose gel electrophoresis of mitochondrial cytochrome B products from amplification with primers 955 and 957. Lanes 1-5 haploid *V. dahliae* (1: 12087, 2: Md71, 3: P14, 4: Md80, 5: Vd128), lanes 6-8 *Verticillium* amphihaploid AFLP group α isolates (6:9010, 7: VdII, 8: 84020), lanes 9-12 *Verticillium* amphihaploid AFLP group β isolates (9: 9802, 10: Md73, 11: 001, 12: 004), lanes 13-14 *V. albo-atrum* (L) (13: STR1, 14: STR3), lanes 15-16 *V. albo-atrum* (NL) (15: VA1, 16: 1974), lane17 *V. nubilum*: 130213, Pc: Primer control, M: molecular marker.

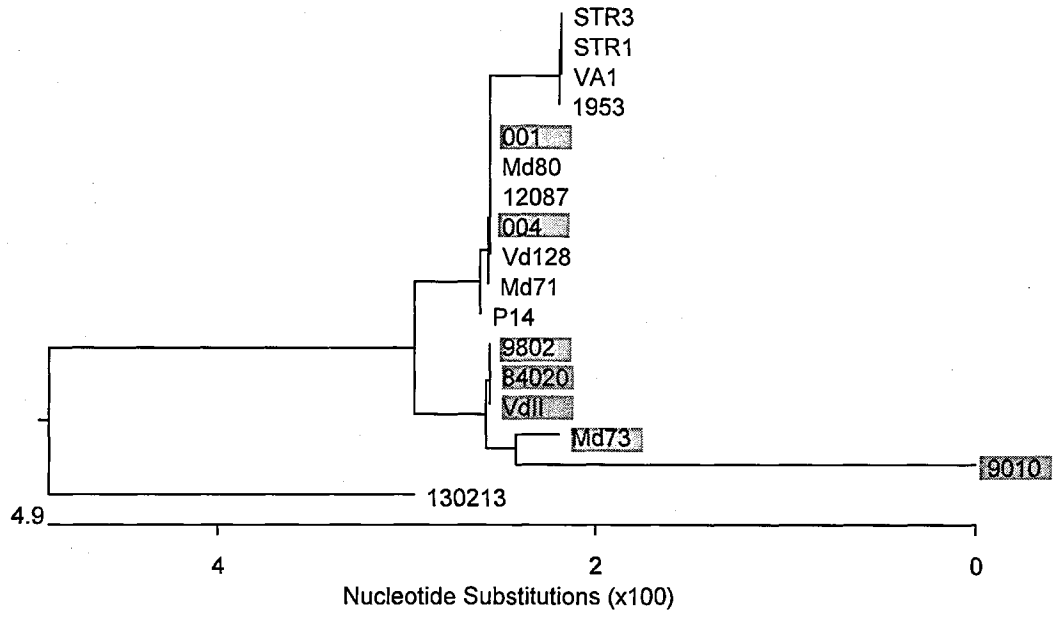

Figure 6.3a. Cladogram of sequence analysis of nucleotides of mitochondrial cytochrome B gene sequences amplified with primers 955 and 957. Letter and numbers refer to isolate names ■ indicates α AFLP group *Verticillium* amphihaploids □ indicates β AFLP group amphihaploids. Isolate 130213 is *V. nubilum.* Generated using ClustalW in MegAlign, Lasergene v 5.07 DNAStar Inc.

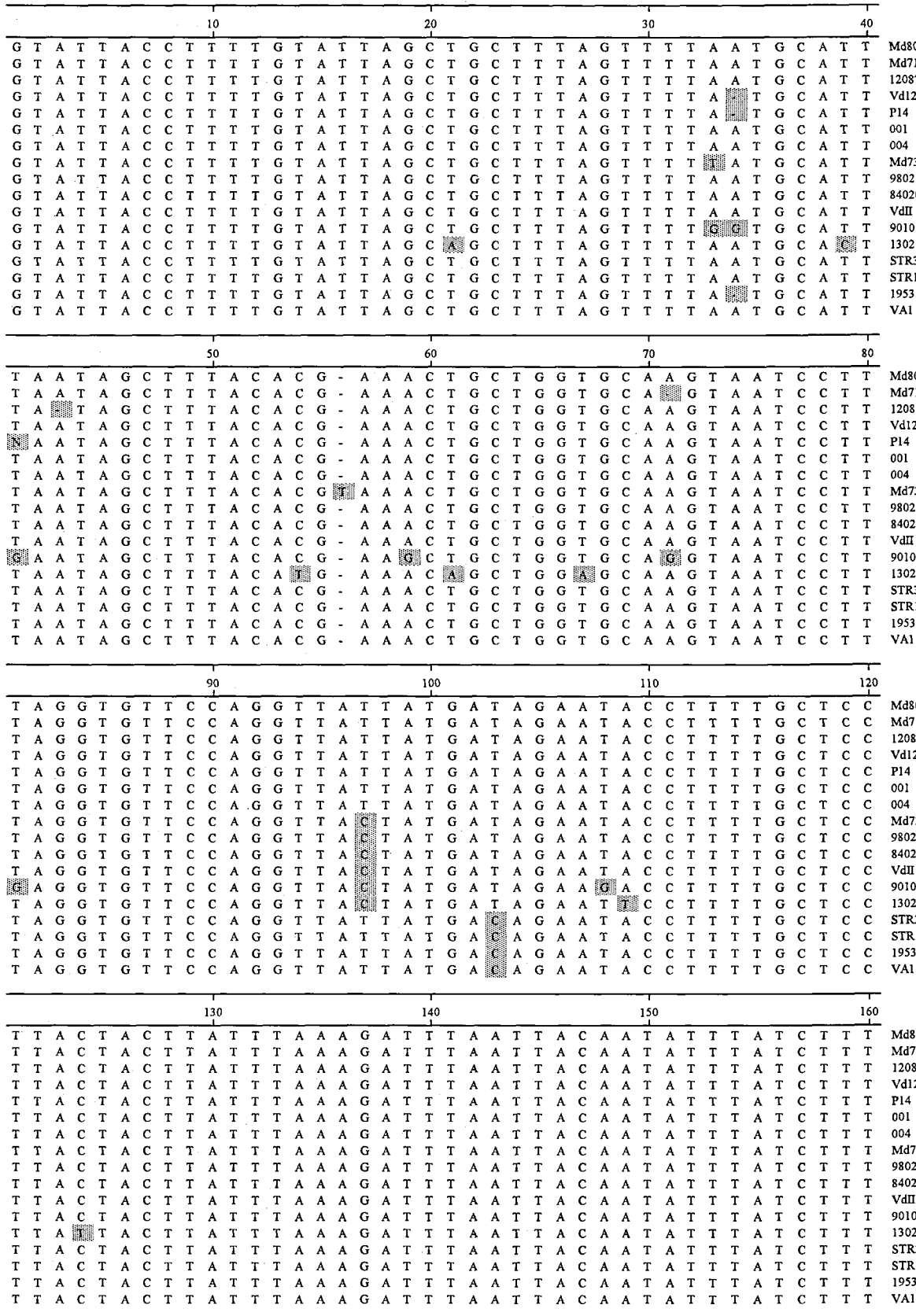

Figure 6.3b. Sequence alignment of mitochondrial cytochrome B gene, with the isolates from Figure 6.3a. Shaded letters indicate main sequence differences. Continued.

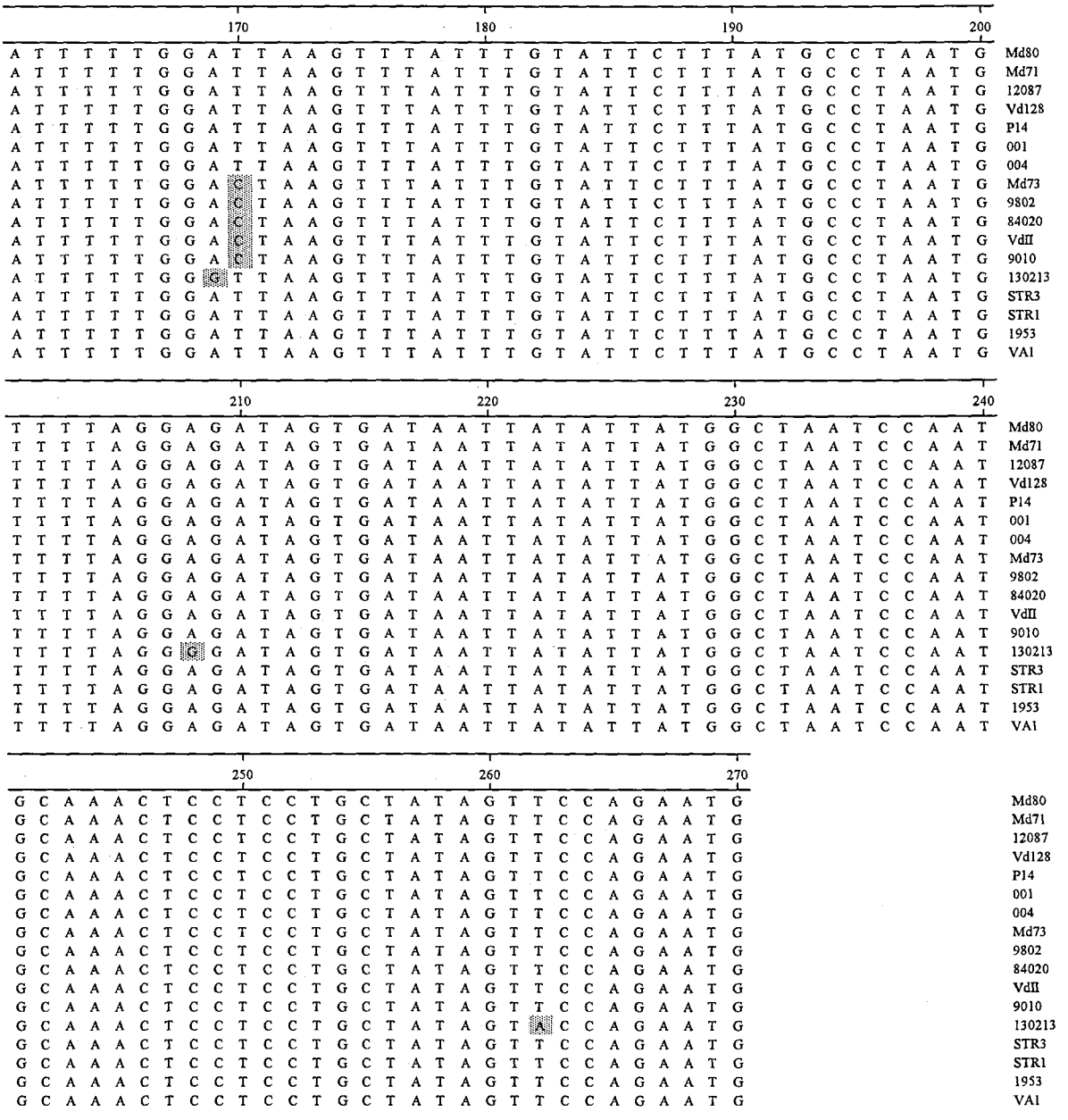

Figure 6.3b. Sequence alignment of mitochondrial cytochrome B gene, with isolates from Figure 6.3a. Shaded letters indicate main sequence differences.

6.3.2 β-tubulin gene

Amplification of a partial sequence of the β-tubulin gene using primers 945 and 946 produced amplicons from all isolates tested (Figure 6.4a). The majority of *V. dahliae* haploid isolates produced single amplicons, however a single isolate, Md71, produced two amplicons. For Md71 the larger of the two products by electrophoresis was of similar size to the single amplicons produced in the other haploid *V. dahliae* isolates, the smaller amplicon by electrophoresis was of similar size to that of amplicons found in amphihaploid isolates of *Verticillium* and an isolate of *V. nubilum*, 130213. All amphihaploid isolates produced at least two amplicons whilst isolates 84020, 9802, Md73, 001, 004 produced three amplicons (Figure 6.4b). In all amphihaploid isolates the largest amplicon was bigger than that of any other isolates tested, however the second amplicon was like the majority of haploid *V. dahliae* isolates and *V. albo-atrum.* The third amplicon seen in some amphihaploid isolates was more variable but smaller and generally similar in size to the second amplicon seen in isolate Md71 and the single amplicon from 130213, an isolate of *V. nubilum.*

Analysis of the sequence of this region of the β-tubulin gene found that this sequence did not produce a single ORF in any frame, and upon further analysis and comparison with other fungal β-tubulin gene sequences deposited on the NCBI database, two introns were identified by comparison with these sequences. These were excised *in silico* and remaining coding sequence spliced to form a single ORF that at the nucleotide and amino acid level displayed high similarity to deposited β-tubulin sequences.

The sequence of the first intron, could be placed into four main clades (Figure 6.5a,b). Of primary significance is that one clade (NL/L) comprised of *V. albo-atrum* isolates only, and that this was distinct from all sequences from haploid *V. dahliae* isolates and *Verticillium* amphihaploids. One clade (A) could be sub-divided into two (A^1 and A^2), this separated haploid *V. dahliae* isolates and 9802 (a *Verticillium* amphihaploid AFLP group β) isolates from amphihaploid isolates from both AFLP group α and β (except 9802). Two small sub-clades (A^3 and A^4) could be formed on the basis of one or two clones, and having no overall effect on the distribution of the isolates between the two major sub-clades. The third clade (B) grouped sequences from amphihaploid *Verticillium* isolates from both AFLP groups, but failed to show clear distinction between both AFLP groups, it however did somewhat divide isolates 9010 and 84020 (AFLP group α amphihaploid isolates from cauliflower hosts in California and *Brassica* hosts in Japan) from oilseed rape isolate VdII (AFLP group α) and 9802, 001, Md73 (AFLP group β isolates). The final clade (D) was distinct from the prior three, and grouped *Verticillium* amphihaploid isolates from both AFLP group α and β together with a single haploid *V. dahliae* isolate, Md71 (Figure 6.5a).

Sequence analysis of the second intron found, also gave four main clades (Figure 6.6a,b), and similarly distinguished isolates of *V. albo-atrum* from all haploid *V. dahliae* and amphihaploid *Verticillium* isolates. One clade (B) grouped only *Verticillium* amphihaploid isolates together of both AFLP α and β groups, a further group (A) clustered amphihaploid *Verticillium* isolates with haploid *V. dahliae*. The final group, D, found by sequence analysis of the second intron, was distinct from the prior three clades, and was as variable within as was the other clades to each other (Figure 6.6b).

As was expected the coding regions were found to be less variable than the introns. Analysis of the of the coding region at the nucleotide level established two main clades (Figure 6.7a,b), the first of which could be weakly sub-divided into three (A, B, NL/L). *V. albo-atrum* was shown to be distinct from the main body of isolates, with one clone (512) as distinct from the rest of the *V. albo-atrum* as they were to *V. dahliae* and the *Verticillium* amphihaploids. Clade B was shown to be quite variable being broadly divided into five. Two sub-clades (B^1 and B^2) comprised entirely of amphihaploid isolates, whilst (B^3 and B^4) comprised amphihaploid *Verticillium* from both AFLP groups and haploid *V. dahliae*. Clade A consisted of clones from haploid *V. dahliae* and amphihaploid *Verticillium* isolates from both AFLP group α and β. Clade C was divergent from A, B and NL/L and comprised few clones from isolates 001 (clone 219) and 84020 (clone 245 and 247). Clade D was highly variable and grouped together clones from haploid *V. dahliae* isolate Md71 and *Verticillium* amphihaploid isolates 84020 (AFLP group α), 001 (AFLP group β) and 9802 (AFLP group β).

Nucleotide sequences were translated into amino acids *in silico* and compared with other fungal β-tubulin proteins. This revealed that the translated sequence bore a high degree of similarity to other fungal β-tubulins deposited on the NCBI database, except that there was an apparent 7aa deletion in the protein sequences of the *Verticillium* isolates tested as compared to, for example, *Ophiostoma pseudotsugae* (Figure 6.8a). Further study of the nucleotide and amino acid sequences found that some of the 'missing' amino acids could be accounted for as part of the second intron sequence removed. However re-insertion of the relevant sequence into the coding sequence pushed the remaining amino acids out of frame. It became apparent that a deletion of a single guanine nucleotide at position 4 within the second intron was found to be the cause of the 7aa deletion, moving the unedited

sequence out of frame at this region; on the basis of this deletion this 'missing' coding sequence was incorporated into the intron but without sequencing the mature mRNA the limits of the intron remain uncertain (Figure 6.8b).

Comparison of the cladograms from the two introns gave some unexpected results. For example, clone 211 according to intron 2 clusters with clone 202 (both clones from isolate 9802 from AFLP group β) however with intron 1 they are divergent from each other falling within different clades (Figure 6.9a). The opposite was true for other clones such as 213 and 216 (both clones from isolate 001 from AFLP group β) (Figure 6.9b). As these apparent inconsistencies may have arisen during sequence comparison, the original sequence data was re-examined to verify these results. Figure 6.9a,b shows that in both these cases this is a true result and not through mismanipulation of the original sequence data.

Combinations of intron 1, intron 2 and nucleotide coding sequences are summarised in Table 6.2 and 6.3. The most frequently occurring intron combination was AA which was found in 25/57 sequences, and the majority of these sequences were found in coding sequence clade A (19/25), the remaining 6 AA intron pairs were placed in clades B (4/25), and C (2/25). The BB intron combination occurred nine times and was usually (8/9) with the B coding sequence. No combinations of introns A and B were found with L/NL or D coding sequence. All *V. albo-atrum* (L and NL) intron combinations occurred with the *V. albo-atrum* (L/NL) coding sequence and the highly divergent sequences represented by clade D in both introns always combined with clade D of the coding sequence. Minority intron combinations AB and BA occurred in 9/57 sequences, with AB the most frequently occurring (7/57) combination. It is assumed that the AA and BB intron pairs, combined

with the A or B coding sequence respectively, represent the parental types for amphihaploid isolates and other combinations of these particular sequences arose through recombination.

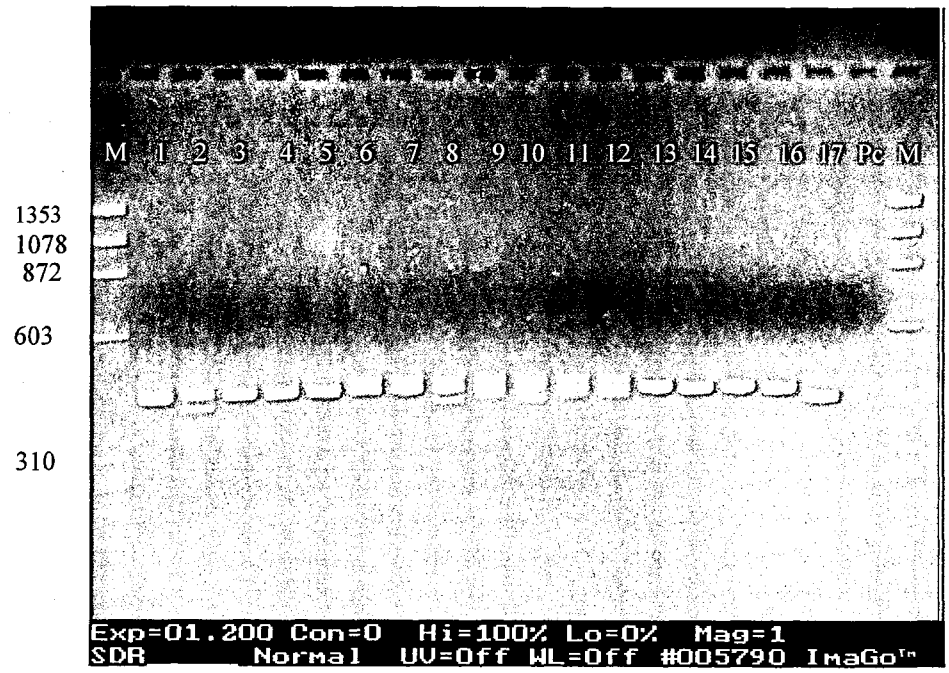

Figure 6.4a. Agarose gel electrophoresis of partial β-tubulin gene products from amplification with primers 945 and 946. Lanes 1-5 haploid *V. dahliae* (1: 12087, 2: Md71, 3: P14, 4: Md80, 5: Vd128), lanes 6-8 *Verticillium* amphihaploid AFLP group α isolates (6:9010, 7: VdII, 8: 84020), lanes 9-12 *Verticillium* amphihaploid AFLP group β isolates (9: 9802, 10: Md73, 11: 001, 12: 004), lanes 13-14 *V. albo-atrum* (L) (13: STR1, 14: STR3), lanes 15-16 *V. albo*-atrum (NL) (15: VA1, 16: 1974), lane17 *V. nubilum*: 130213, Pc: Primer control, M: molecular marker.

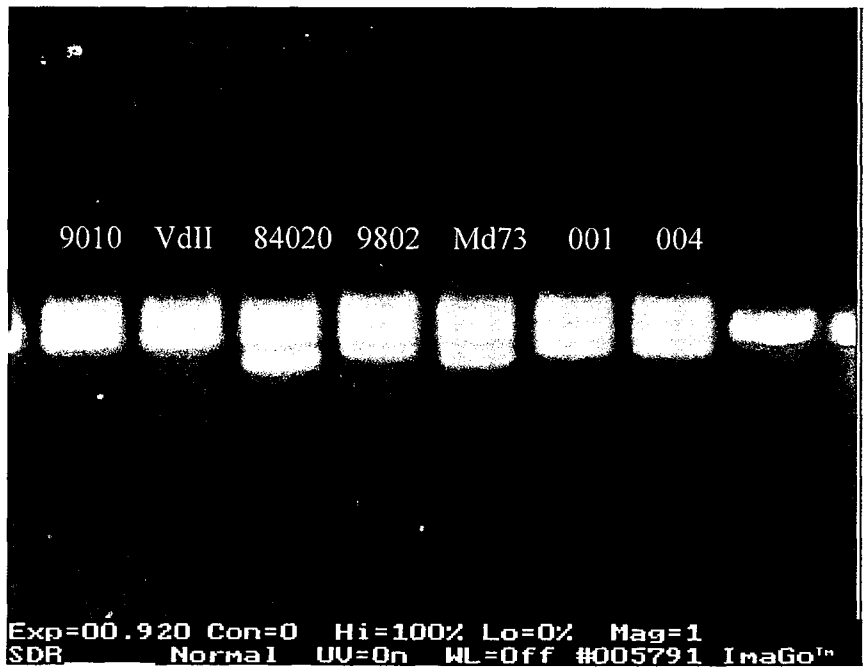

Figure 6.4b. Enlarged photograph of agarose gel electrophoresis of partial β-tubulin gene products from amplification with primers 945 and 946. Multiple amplification products produced by *Verticillium* amphihaploid isolates.

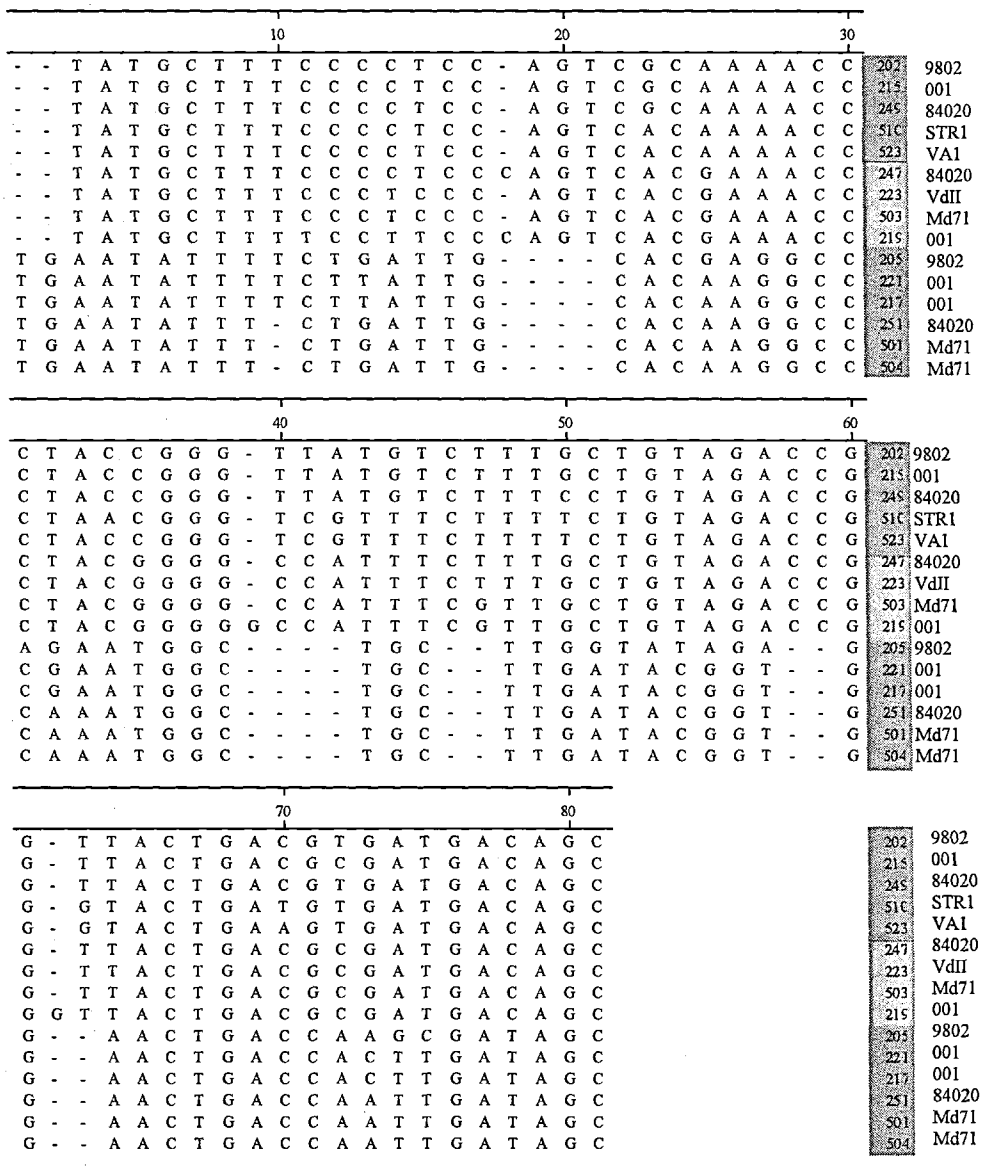

Figure 6.5a. Sequence alignment of Intron 1, with typical representative isolates from each clade of Figure 6.5b. ☐ clade B, ☐ clade L/NL; ☐ clade A; ☐ clade D. Shaded numerals are clone identifiers, unshaded letters and numbers are isolate names (Table 6.1)

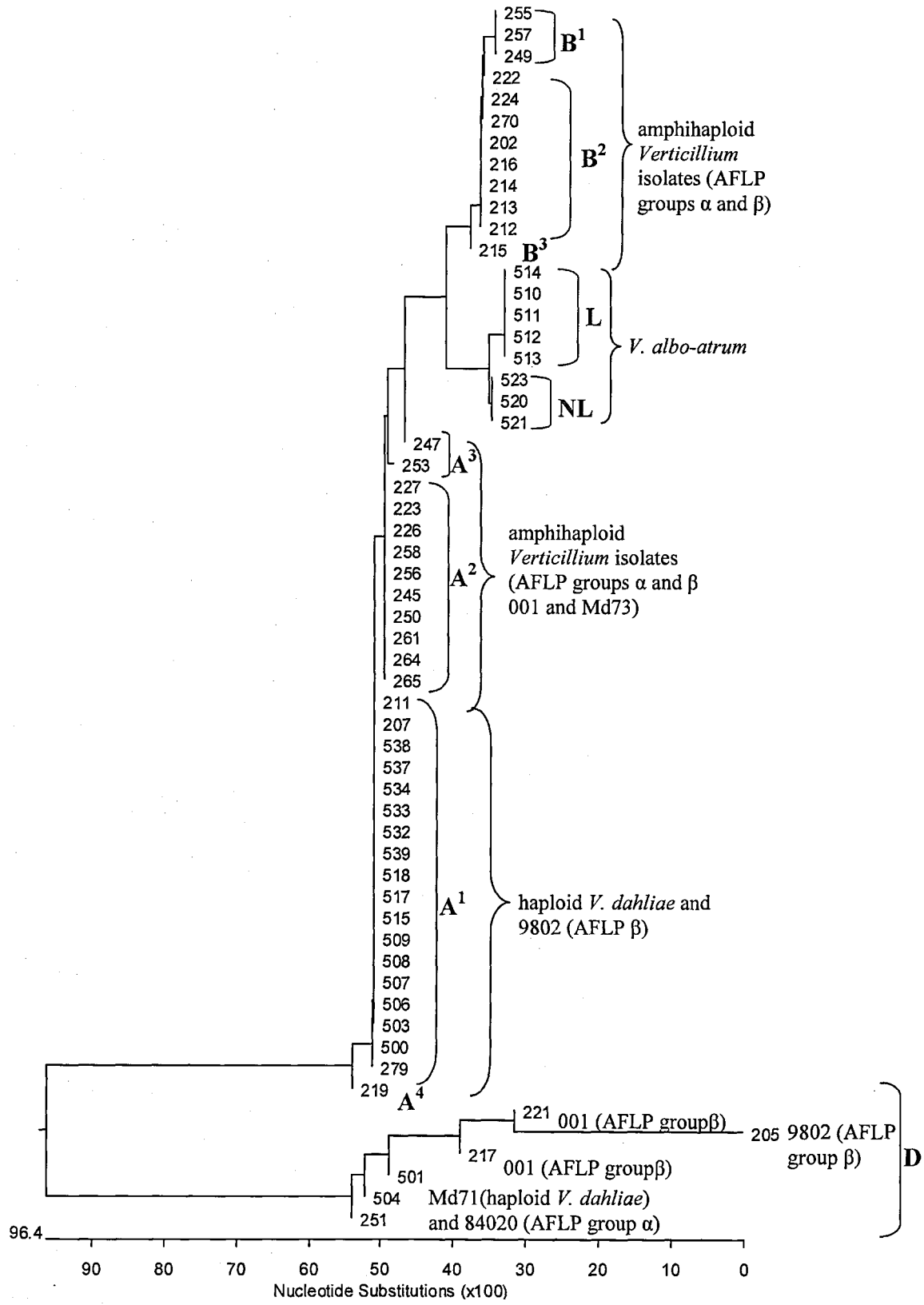

Figure 6.5b. Cladogram of sequence analysis of intron 1 of partial β-tubulin sequence amplified with primers 945/946. Numeral at the end of branches are clone identifiers. Multiple clones of each isolate were sequenced (Table 6.1). Coloured brackets represent intron groups, refer to Table 6.1. Generated using ClustalW in MegAlign, Lasergene v 5.07 DNAStar Inc.

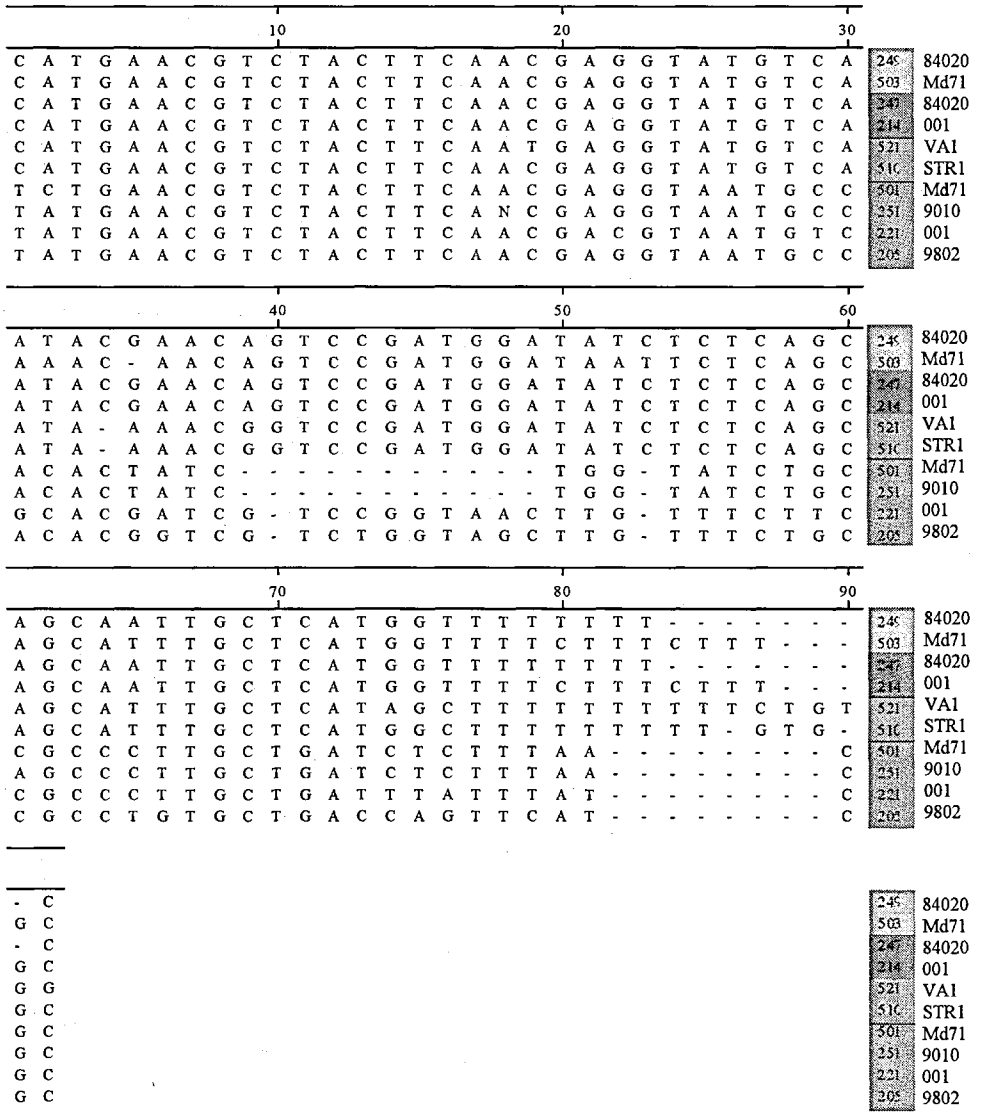

Figure 6.6a. Sequence alignment of Intron 2, with typical representative isolates from each clade of Figure 6.5b. □clade B, □ clade L/NL; □ clade A; □clade D. Shaded numerals are clone identifiers, unshaded letters and numbers are isolate names (Table 6.1).

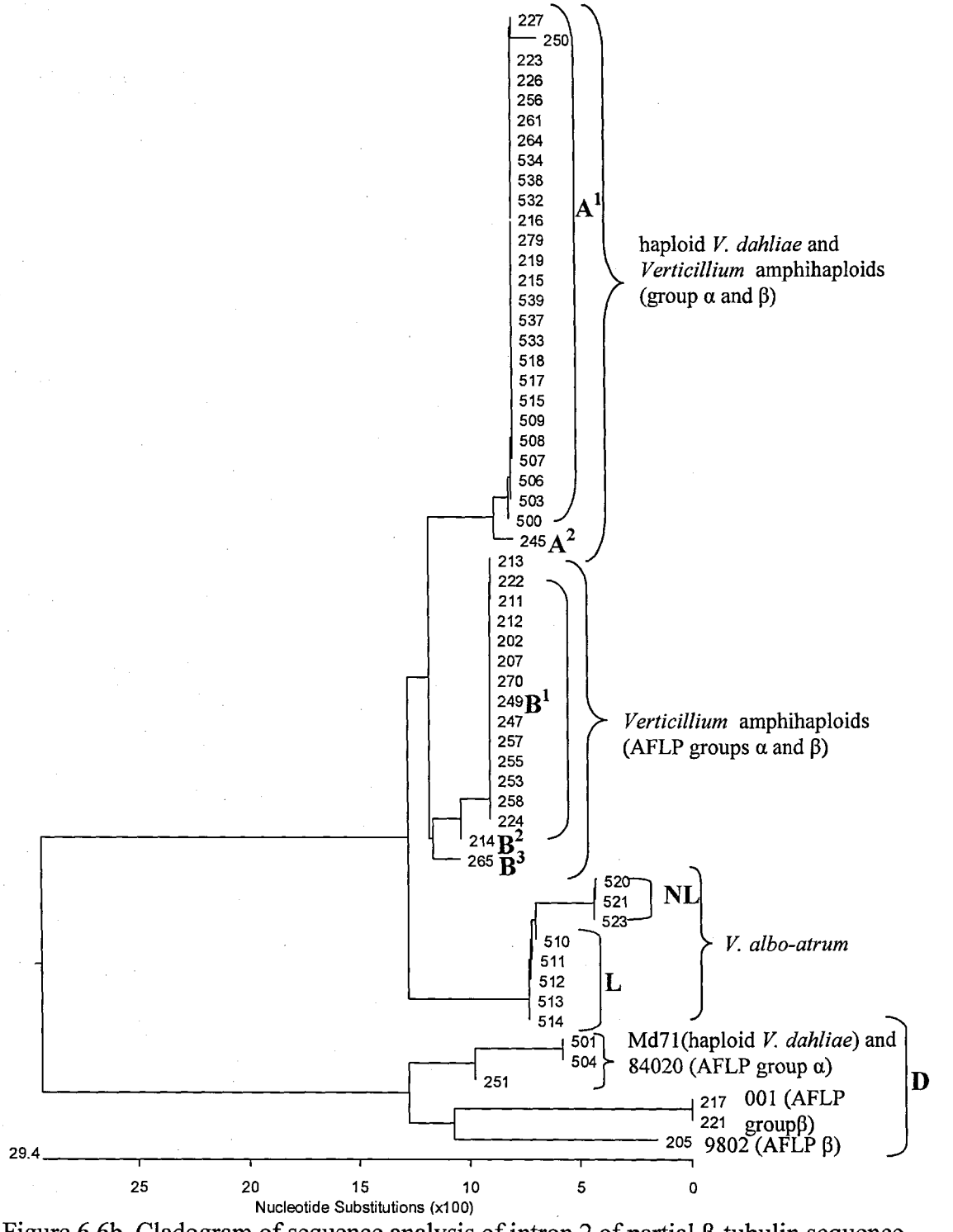

Figure 6.6b. Cladogram of sequence analysis of intron 2 of partial β-tubulin sequence amplified with primers 945/946. Numeral at the end of branches are clone identifiers. Multiple clones of each isolate were sequenced (Table 6.1) Coloured brackets and letters represent intron groups refer to Table 6.1. Generated using ClustalW in MegAlign, Lasergene v 5.07 DNAStar Inc.

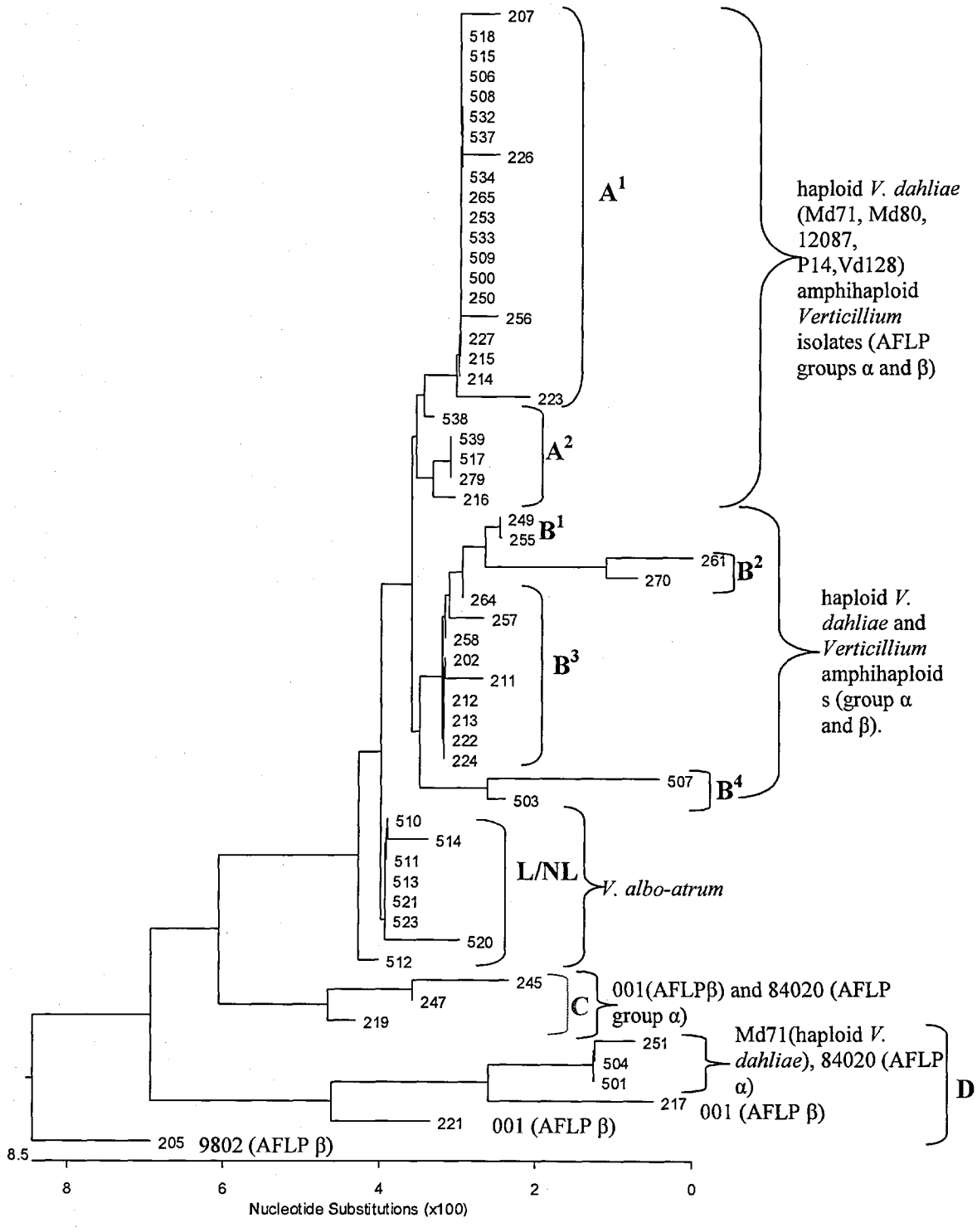

Figure 6.7a. Cladogram of sequence analysis of nucleotides of coding sequence of partial β-tubulin sequence amplified with primers 945/946. Numeral at the end of branches are clone identifiers. Multiple clones of each isolate were sequenced (Table 6.1). Generated using ClustalW in MegAlign, Lasergene v 5.07 DNAStar Inc.

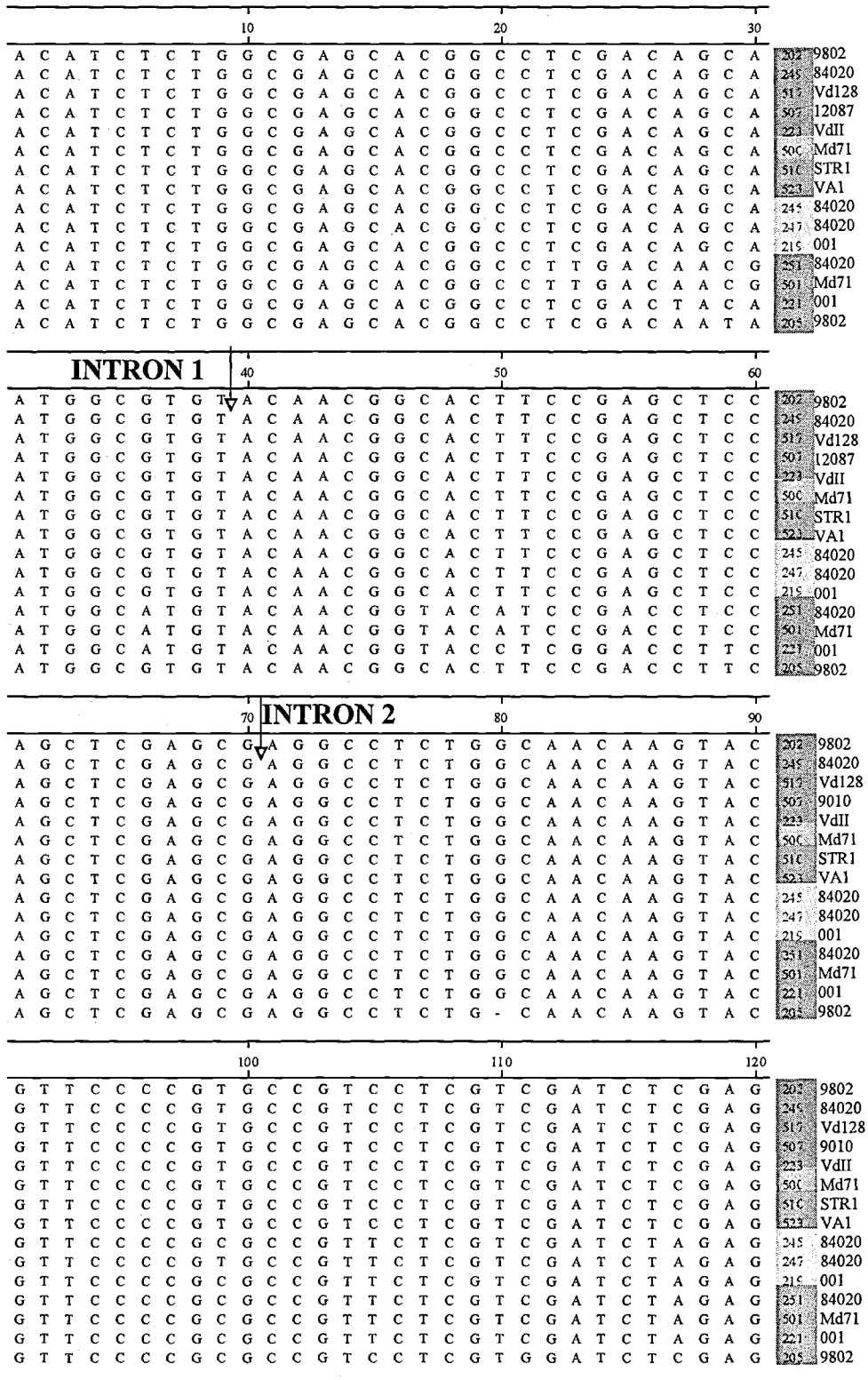

Figure 6.7b. Sequence alignment of nucleotide coding sequence with introns removed, with typical representative isolates from each clade of Figure 6.7a. ☐clade B, ☐ clade L/NL; ☐ clade A; ☐ clade D; clade C . Shaded numerals are clone identifiers, unshaded numbers and letters are isolate names (Table 6.1). Continued.

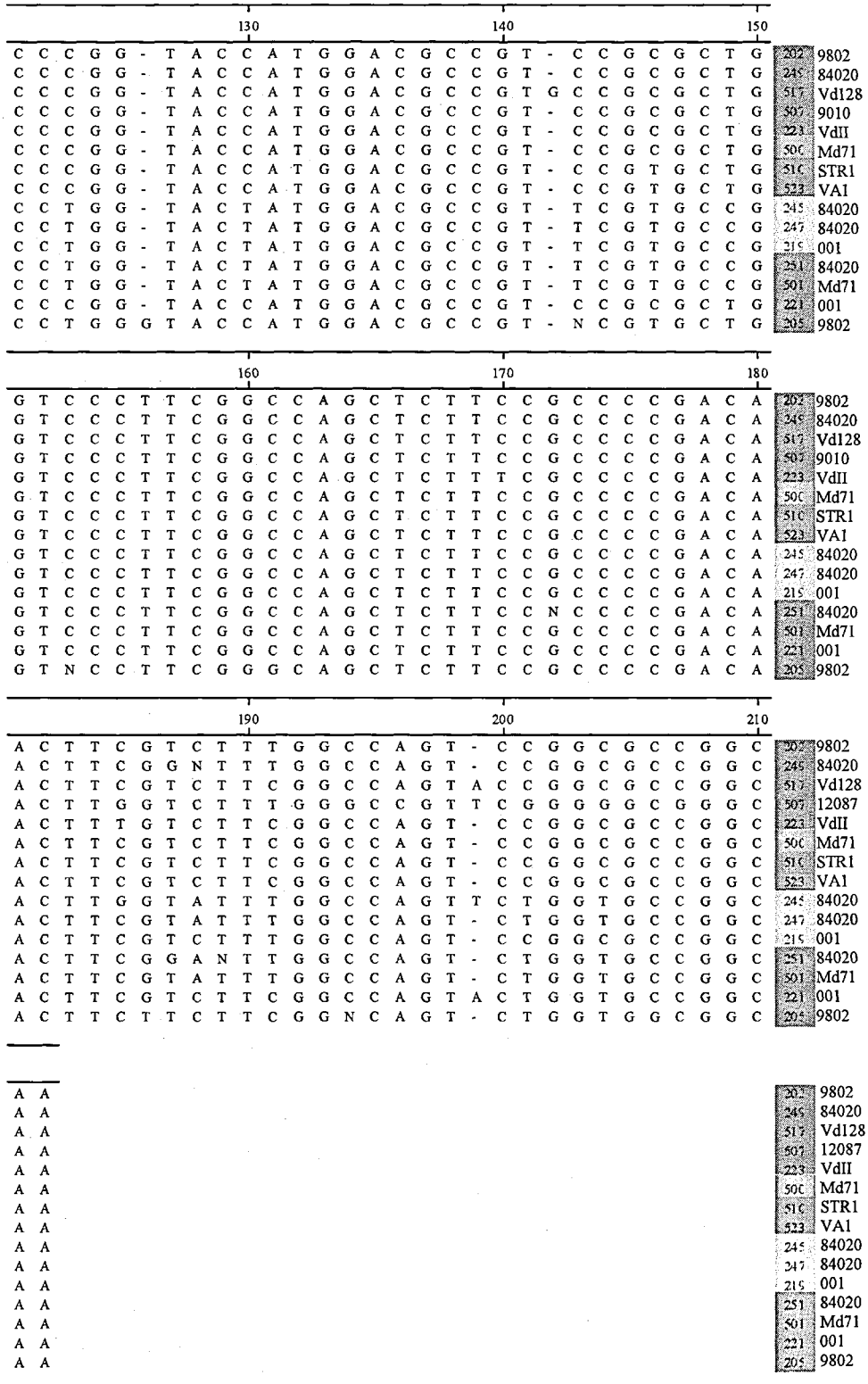

Figure 6.7b. Sequence alignment of nucleotide coding sequence with introns removed, with typical representative isolates from each clade of Figure 6.7a clade B, clade L/NL; clade A; clade D clade C . Shaded numerals are clone identifiers, unshaded letters and numbers are isolate names (Table 6.1).

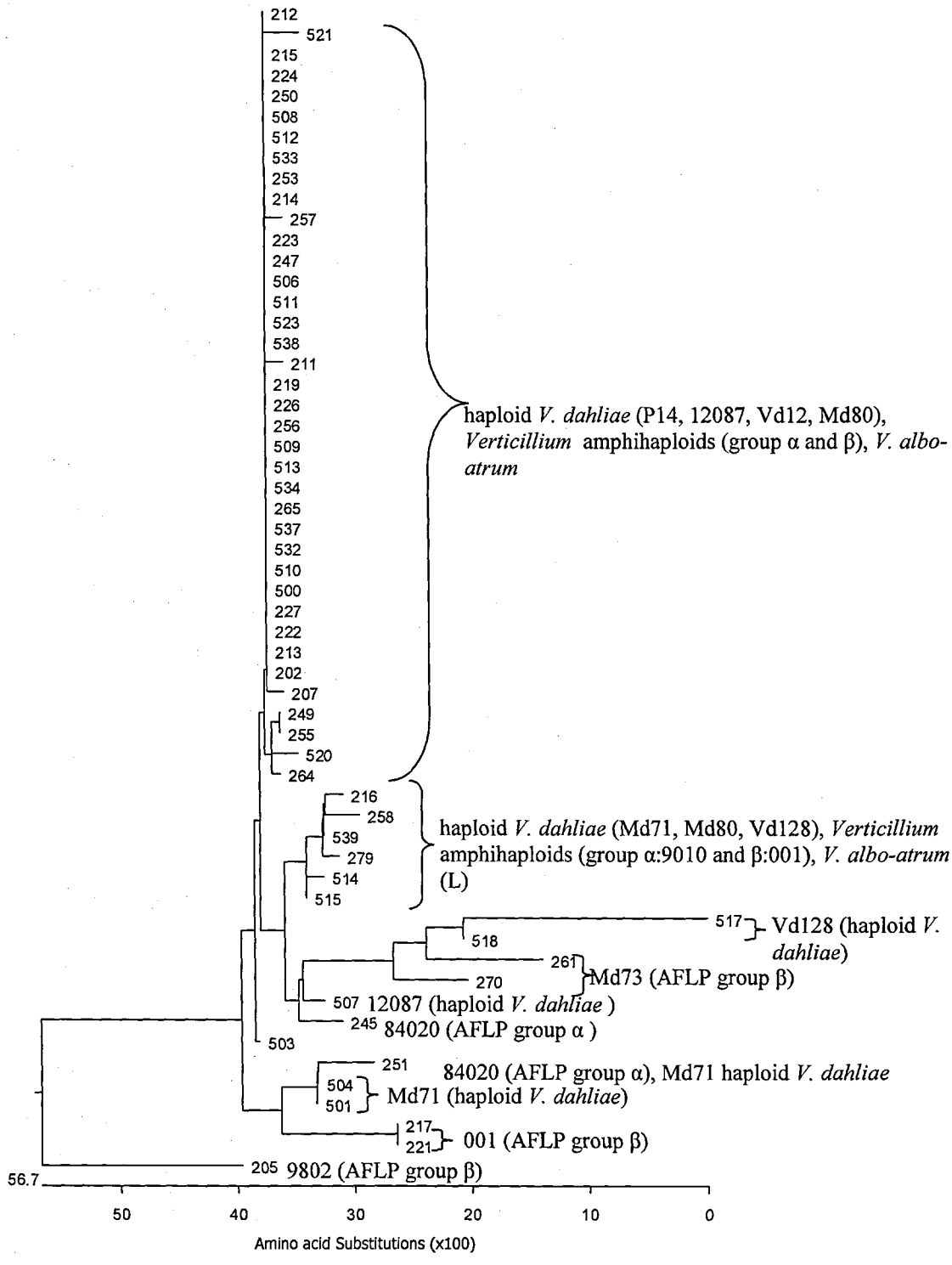

Figure 6.7c. Cladogram of sequence analysis of amino acids of coding sequence of partial β-tubulin sequence amplified with primers 945/946. Numerals at the end of branches are clone identifiers. Multiple clones of each isolate were sequenced (Table 6.1)Generated using ClustalW in MegAlign, Lasergene v 5.07 DNAStar Inc.

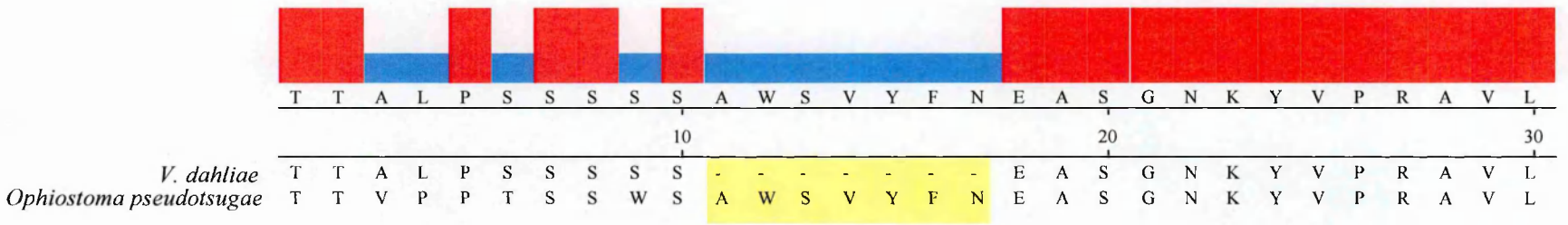

Figure 6.8a

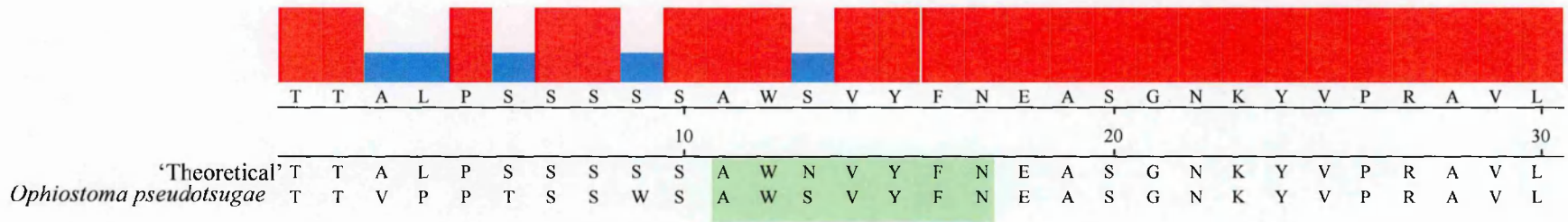

Figure 6.8b

Figure 6.8a. Alignment of partial amino acid sequences from a typical *V. dahliae* β-tubulin sequence and *Ophiostoma pseudotsugae* (GenBank accession number: AAS55936). Indicates the region where due to a single base pair deletion in plant pathogenic *Verticillium* sp. a 7aa region is lost.

Figure 6.8b. Alignment of partial amino acid sequences from a theoretical *Verticillium* β-tubulin sequence with a single added 'G' nucleotide and *Ophiostoma pseudotsugae* (GenBank accession number: AAS55936). Indicates where if the nucleotide is replaced, and the position of the intron adjusted, in plant pathogenic *Verticillium* the 7aa region is restored.

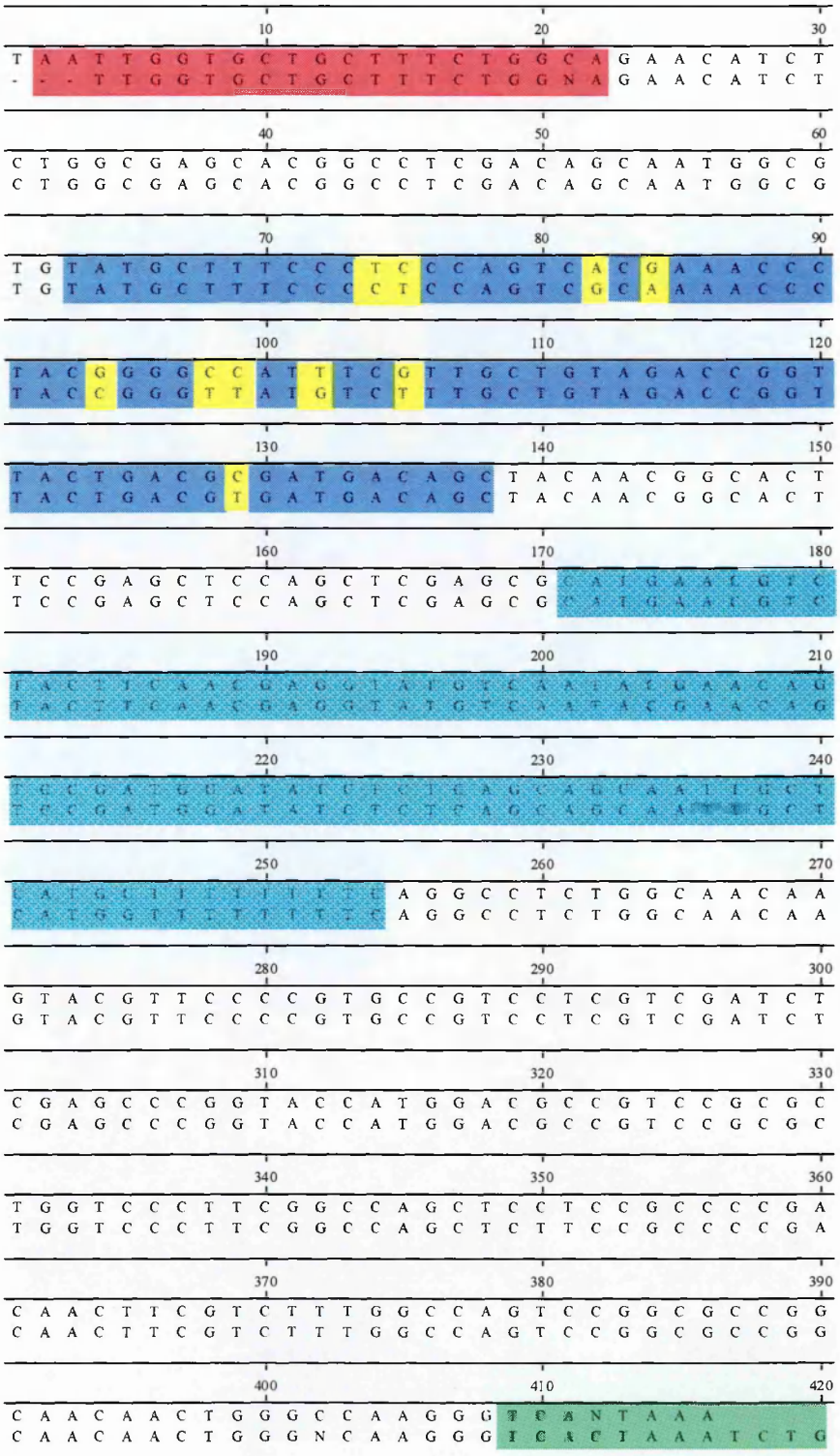

Figure 6.9a. Alignment of unedited sequence of clones 211 and 202, both from *Verticillium* amphihaploid AFLP group β isolate 9802 . ■ and ■ are the forward and reverse primer, ■ indicates the position of the first intron and ■ highlights the sequence differences within the intron. ■ Indicates the position of the second intron, with no sequence differences.

211
202

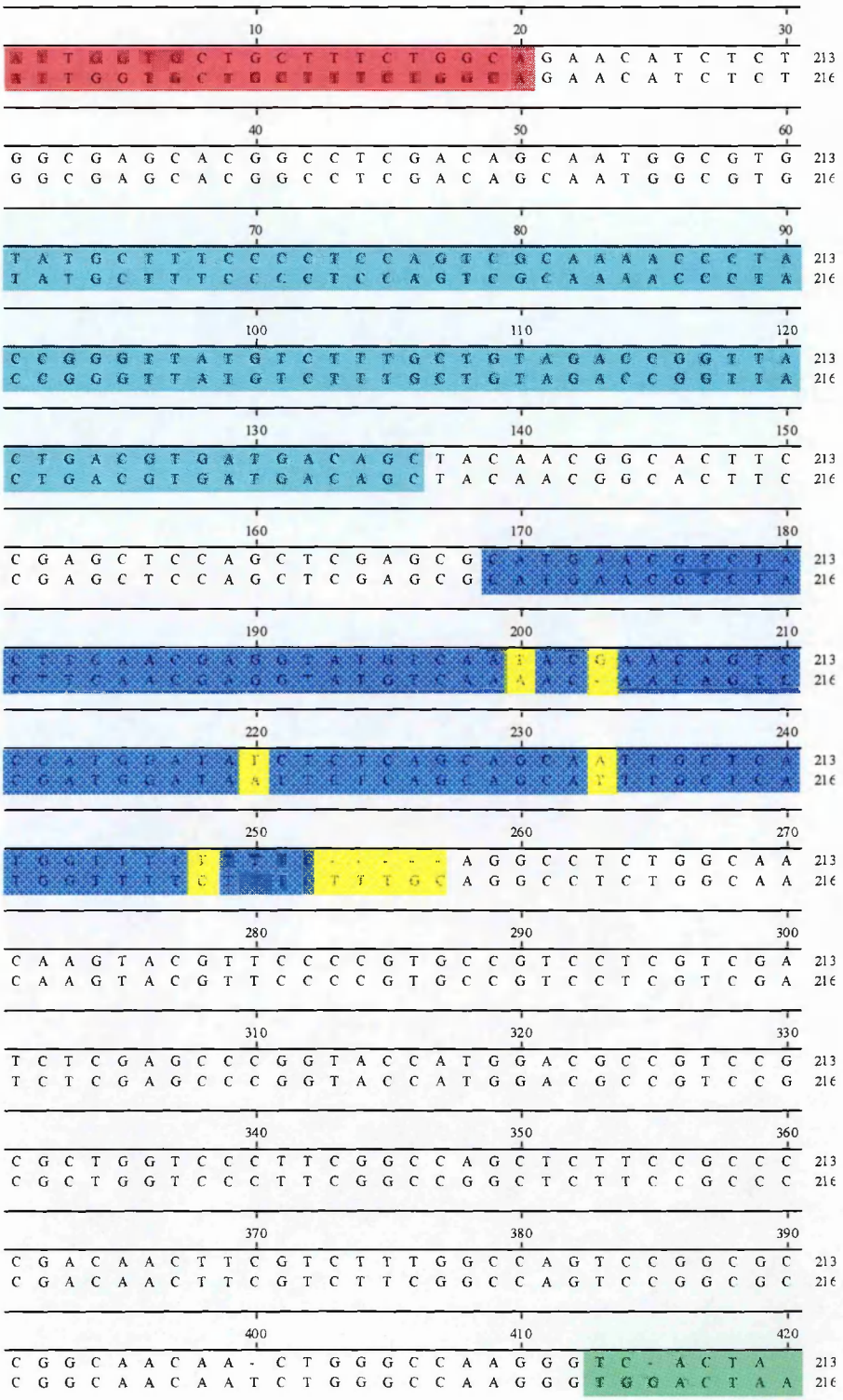

Figure 6.9b. Alignment of unedited sequence of clones 213 and 216, both from *Verticillium* amphihaploid AFLP group β isolate 9802 . and are the forward and reverse primer, indicates the position of the second intron and highlights the sequence differences within the intron. indicates the position of the first intron with no sequence differences.

Isolate	Clone	Intron 1[§]	Intron 2[*]	CDS[‡]
STR1	510	L	L	L/NL
	511	L	L	L/NL
	512	L	L	L/NL
	513	L	L	L/NL
	514	L	L	L/NL
VA1	520	NL	NL	L/NL
	521	NL	NL	L/NL
	523	NL	NL	L/NL
12087	506	A^1	A^1	A^1
	507	A^1	A^1	B^4
	508	A^1	A^1	A^1
	509	A^1	A^1	A^1
Md71	279	A^1	A^1	A^2
	500	A^1	A^1	A^1
	501	D	D	D
	503	A^1	A^1	B^4
	504	D	D	D
P14	532	A^1	A^1	A^1
	533	A^1	A^1	A^1
	534	A^1	A^1	A^1
Md80	537	A^1	A^1	A^1
	538	A^1	A^1	A^1
	539	A^1	A^1	A^2
Vd128	515	A^1	A^1	A^1
	517	A^1	A^1	A^2
	518	A^1	A^1	A^1
VdII	222	B^2	B^1	B^3
	223	A^2	A^1	A^1
	224	B^2	B^1	B^3
	226	A^2	A^1	A^1
	227	A^2	A^1	A^1
9010	**253**	**A^3**	**B^1**	A^1
	255	B^1	B^1	B^1
	256	A^2	A^1	A^1
	257	B^1	B^1	B^3
	258	**A^2**	**B^1**	B^3
84020	245	A^2	A^2	C
	247	**A^3**	**B^1**	C
	249	B^1	B^1	B^1
	250	A^2	A^1	A^1
	251	D	D	D
9802	202	B^2	B^1	B^3
	205	D	D	D
	207	**A^1**	**B^1**	A^1
	211	**A^1**	**B^1**	B^3
001	212	B^2	B^1	B^3
	213	B^2	B^1	B^3

001 continued	214	B^2	B^2	A^1
	215	**B^3**	**A^1**	A^1
	216	**B^2**	**A^1**	A^2
	217	D	D	D
	219	A^4	A^1	C
	221	D	D	D
Md73	261	A^2	A^1	B^2
	264	A^2	A^1	B^3
	265	**A^2**	**B^3**	A^1
	270	B^2	B^1	B^2

Table 6.2. Summary Table of isolate, clones and β-tubulin intron combinations found. highlights intron minority combinations. § refer to Figure 6.5b, letters refer to clades/intron type; ж refer to Figure 6.6b, letters refer to clades/intron type; £ refers to 6.7a, letters refer to clades.

Intron 1/2	**Coding sequence**					
	A	**B**	**C**	**D**	**L/NL**	**Total**
AA	**19**	**4**	**2**	**0**	**0**	**25**
AB	**3**	**2**	**1**	**0**	**0**	**6**
BA	**2**	**0**	**0**	**0**	**0**	**2**
BB	**1**	**9**	**0**	**0**	**0**	**10**
DD	**0**	**0**	**0**	**6**	**0**	**6**
LL	**0**	**0**	**0**	**0**	**5**	**5**
NLNL	**0**	**0**	**0**	**0**	**3**	**3**
Total	**25**	**15**	**3**	**6**	**8**	**57**

Table 6.3. Summary Table of intron1/intron2/coding sequence combinations

6.3.3 Southern analysis of β-tubulin gene

Figures 6.10a and 6.10b show the fluorograms of the hybridisation between the β-tubulin gene probe and genomic DNA digested with either *Pvu*I or *Nru*I. The β-tubulin gene probe hybridised to all isolates tested, except isolate 1988 digested with *Nru*I. The reason for this failure with 1988 is unknown as the probe hybridised to this isolate when digested with *Pvu*I.

Haploid *V. dahliae* isolates 12087, P14, Md80, Vd128 (lane 2-5, Figure 6.10a and 6.10b) all gave a single band of similar size (more than 11 kbp) indicating that these isolates have at least a single copy of the β-tubulin gene. Isolate Md71, a haploid *V. dahliae*, gave two bands (more than 11 kbp, 5-6 kbp) suggesting that in this isolate there are at least two copies of the β-tubulin gene. However, the blot of genomic DNA digested with *Pvu*I (Figure 6.10b) did not give bands of equal strength for isolate Md71, suggesting that the larger band that corresponded in size to the other haploid *V. dahliae* isolates may have been an artefact. This possibility was discounted as the blot of genomic DNA digested with *Nru*I gave two bands of equal strength (more than 11 kbp, approximately 9 kbp) and this isolate does appear to carry two copies of this gene when tested by PCR (Figure 6.4 a,b) and by Southern analysis (Figure 6.10a, b).

V. albo-atrum isolates 1974, VA1 and STR1 gave single bands in both blots (*Nru*I approximately 5 kbp, *Pvu*I more than 11 kbp) indicating that there was at least one copy of this gene in this species. *V. nubilum* isolate 130213 also gave a single band in both blots again (*Nru*I 10-11 kbp, *Pvu*I more than11 kbp) indicating that there is at least one copy of the β-tubulin gene in this isolate. Isolates 1988 and 151 of *V. tricorpus* and *V. albo-atrum* (GpII) produced different results in both blots. As mentioned, digestion of genomic DNA

of isolate 1988 with *Nru*I did not give any bands of any size, however *Pvu*I produced three bands (more than 11kb, approximately 11 kbp, 5-6 kbp), but with decreasing intensities from the larger band through to the smallest of the three. Isolate 151 gave two bands when digested with *Pvu*I (more than 11 kbp, 5-6 kbp), but a single band when digested with *Nru*I (approximately 3 kbp).

Of the amphihaploid isolates, VdII produced a single band with both enzymes, however with *Pvu*I either accidentally less DNA was loaded or the probe hybridised poorly for some other reason as the band was not easily visualised. 9010 gave a single band with *Pvu*I and two with *Nru*I. Isolate 84020 gave two bands in each blot.

From the AFLP group β isolates, 9802 gave two products with digestion by *Nru*I, and one with *Pvu*I. Md73 gave two and three bands with *Pvu*I and *Nru*I respectively. 001 and 004, both horseradish isolates from Illinois, gave three bands when digested with *Nru*I; with *Pvu*I, the probe did not hybridise to 001 but gave three bands for isolate 004.

	haploid *V. dahliae*					amphihaploid *Verticillium*							*V. albo-atrum, V. nubilum, V. tricorpus, V. albo-atrum* (GpII)					
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**
***Nru*I**	2	1	1	1	1	1	2	2	2	3	3	3	1	1	1	1	?	2
***Pvu*I**	2	1	1	1	1	1	2	2	1	2	?	3	1	1	1	1	3	1
Copy number?	2	1	1	1	1	1	2	2	2	3	3	3	1	1	1	1	3	2

Table 6.4. Summary Table of minimum copy number of β-tubulin gene 1:Md71, 2: 12087, 3: P14, 4: Md80, 5: Vd128, 6: VdII, 7: 9010, 8: 84020, 9: 9802, 10: Md 73, 11: 001, 12: 004, 13: 1974, 14: VA1, 15: STR1, 16: 130213, 17: 1988, 18: 151 AFLP group α isolates AFLP group β isolates.

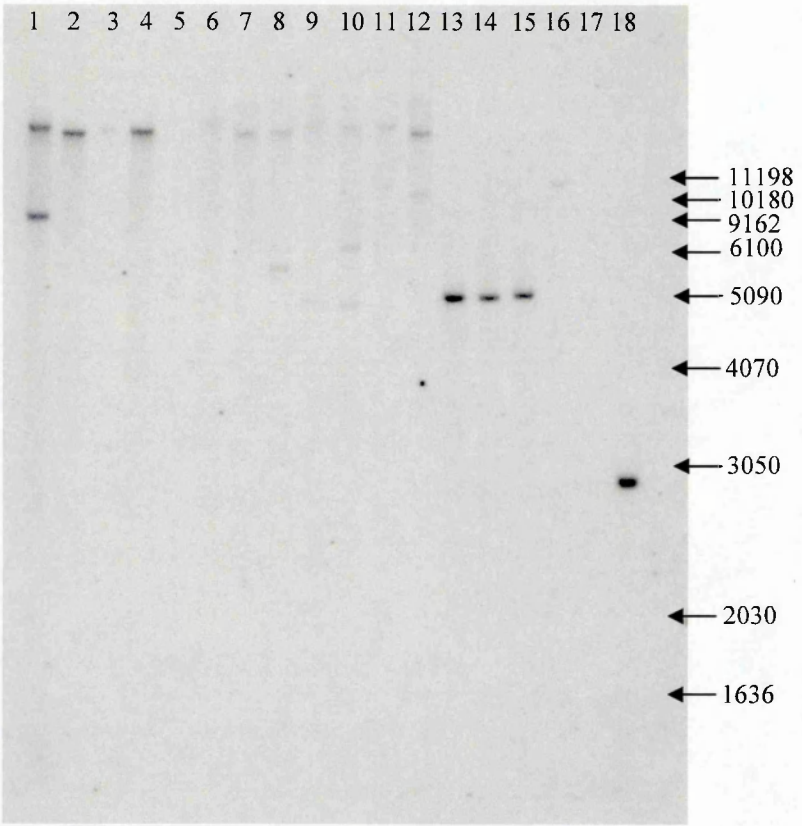

Figure 6.10a. Southern blot of genomic DNA digested with *Nru*I probed with β-tubulin gene probe. Lanes 1-5 haploid *V. dahliae* (1: Md71, 2: 12087, 3: P14, 4: Md80, 5: Vd128), lanes 6-8 *Verticillium* amphihaploid AFLP group α isolates (6:VdII, 7: 9010, 8: 84020), lanes 9-12 *Verticillium* amphihaploid AFLP group β isolates (9: 9802, 10: Md73, 11: 001, 12: 004), lanes 13-14 *V. albo-atrum* (NL) (13: 1974, 14: VA1), lanes 15 *V. albo-atrum* (L) (15: STR1), lane 16 *V. nubilum*: 130213, lane 17 *V. tricorpus* (17:1988), lane 18: *V. albo-atrum* (GpII) (18:151).

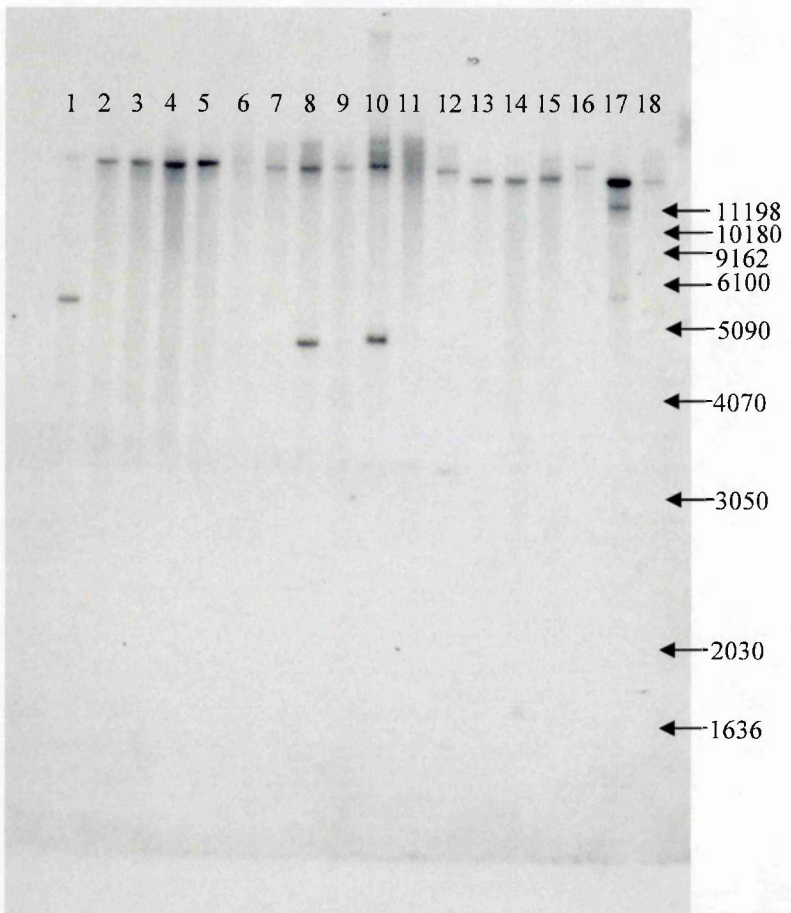

Figure 6.10b. Southern blot of genomic DNA digested with *Pvu*I probed with β-tubulin gene Lanes 1-5 haploid *V. dahliae* (1: Md71, 2: 12087, 3: P14, 4: Md80, 5: Vd128), lanes 6-8 *Verticillium* amphihaploid AFLP group α isolates (6:VdII, 7: 9010, 8: 84020), lanes 9-12 *Verticillium* amphihaploid AFLP group β isolates (9: 9802, 10: Md73, 11: 001, 12: 004), lanes 13-14 *V. albo-atrum* (NL) (13: 1974, 14: VA1), lanes 15 *V. albo-atrum* (L) (15: STR1), lane 16 *V. nubilum*: 130213, lane 17 *V. tricorpus* (17:1988), lane 18: *V. albo-atrum* (GpII) (18:151).

6.3.4 5*S* rRNA gene intergenic region

PCR amplification from the 5*S* rRNA gene using the 'outward facing' primers 846 and 847 produced specific products in haploid *V. dahliae* and amphihaploid *Verticillium* isolates, thus indicating that there were at least two 5*S* rRNA genes in close proximity to each other separated by an intergenic region (IGR) (Figure 6.11a). PCR amplification of the 5*S* rRNA IGR using these primers produced single amplicons with all haploid *V. dahliae* isolates tested; *V. albo-atrum* isolates produced weak and variable amplicons, as did isolates of *V. nubilum*, *V. albo-atrum* (Gp II) or *V. tricorpus* (data not shown for *V. albo-atrum* GpII or *V. tricorpus*). However, these weak amplicons were somewhat consistent within groups *e.g.* both Lucerne isolates of *V. albo-atrum* and Non-Lucerne isolates. *Verticillium* amphihaploid isolates all produced two amplicons (Figure 6.11b) with the smaller of the amplicons of similar size to that of the haploid *V. dahliae* isolates. The larger amplicon did not have any corresponding sized amplicon in any haploid isolate tested. Sequence analysis showed that this length polymorphism was accounted for mainly by three insertions/deletions of 4 bp, 9 bp and 12 bp that were separated by two conserved regions of 30 bp and 4 bp (Figure 6.12; 6.13). These insertions/deletions were conserved across all amphihaploid isolates, and within all sequence variants of this larger amplicon.

Further sequence analysis showed that at this locus there were 6 main clades (Figure 6.14). Two clades contained only haploid isolates (12087/Md71 and P14/Vd128). The haploid isolate Md80 fell into a third clade with the AFLP group β isolates 001/004. Two further clades contained the AFLP group β isolates (Md73 and 9802) or AFLP group α isolates (VdII, 9010, 84020). The final clade contained all the longer sequences from amphihaploid isolates in both α and β AFLP groups with no apparent subdivision within or between AFLP groups.

Figure 6.11a. Agarose gel electrophoresis of 5*S* rRNA gene IGR products from amplification with primers 846 and 847. Lanes 1-5 haploid *V. dahliae* (1: 12087, 2: Md71, 3: P14, 4: Md80, 5: Vd128), lanes 6-8 *Verticillium* amphihaploid AFLP group α isolates (6:9010, 7: VdII, 8: 84020), lanes 9-12 *Verticillium* amphihaploid AFLP group β isolates (9: 9802, 10: Md73, 11: 001, 12: 004), lanes 13-14 *V. albo-atrum* (L) (13: STR1, 14: STR3), lanes 15 *V. albo*-atrum (NL) (15: VA1 16: 1974), lane 17 *V. nubilum*: 130213 Pc: primer control, M: molecular marker.

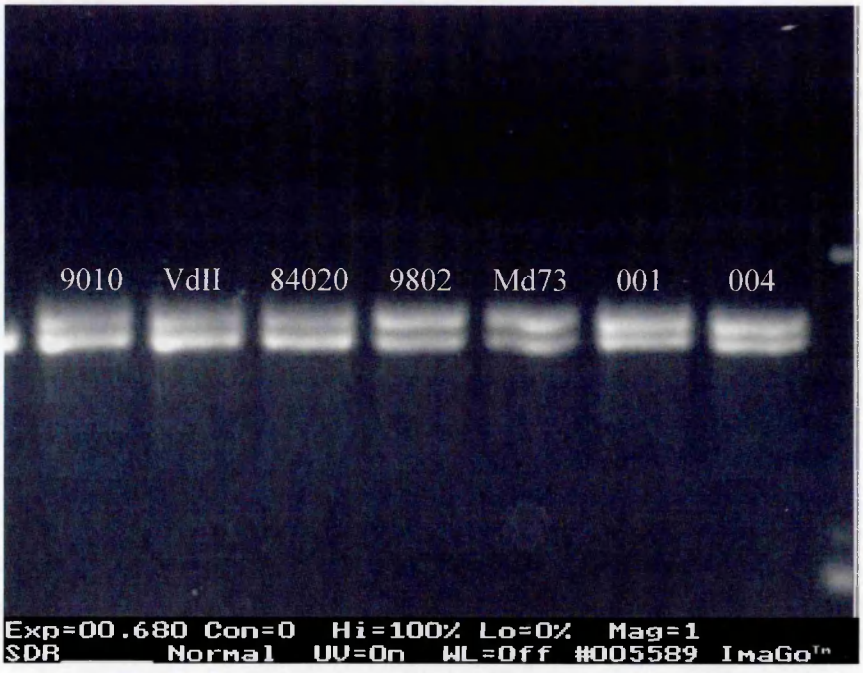

Figure 6.11b. Enlarged photograph of agarose gel electrophoresis of 5*S* rRNA gene IGR products from amplification with primers 846 and 847.

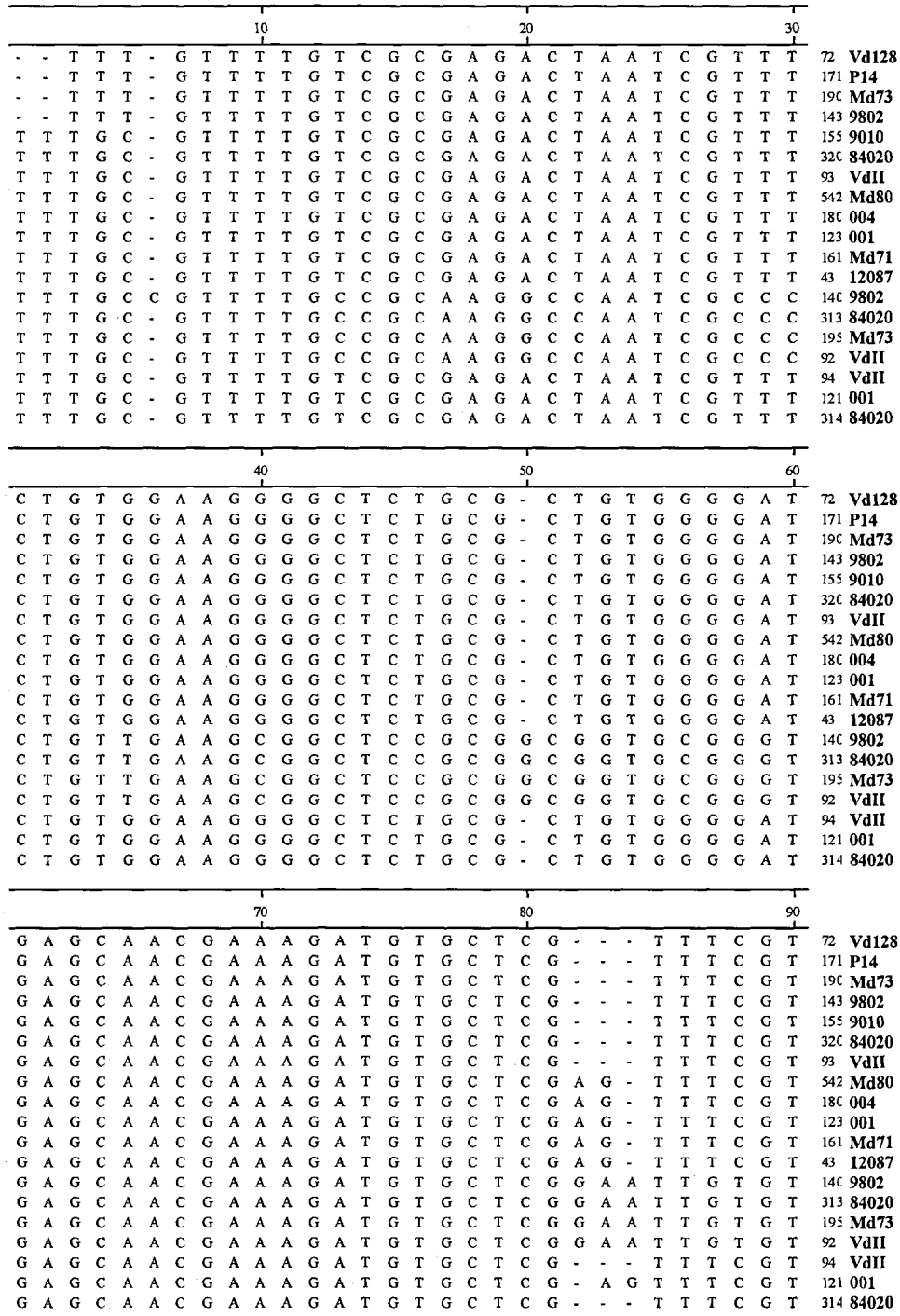

Figure 6.12. Alignment of 5*S* rRNA IGR typical sequences from each clade of Figure 6.14. Numbers are clones identifiers. Letter and numbers in bold are isolate names (See Table 6.1). Continued.

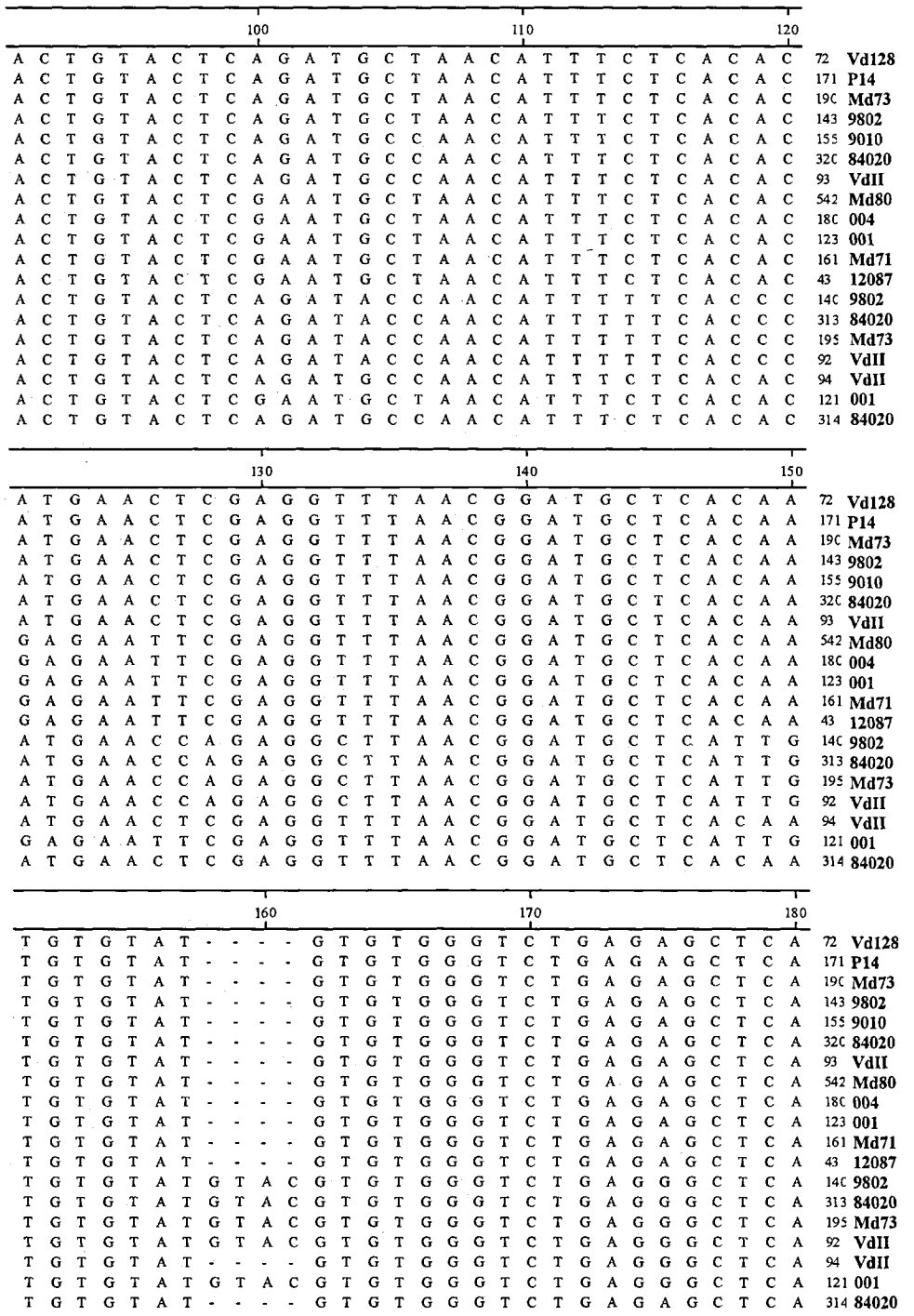

Figure 6.12. Alignment of 5*S* rRNA IGR typical sequences from each clade of Figure 6.14. Numbers are clones identifiers. Letter and numbers in bold are isolate names. (See Table 6.1). Continued.

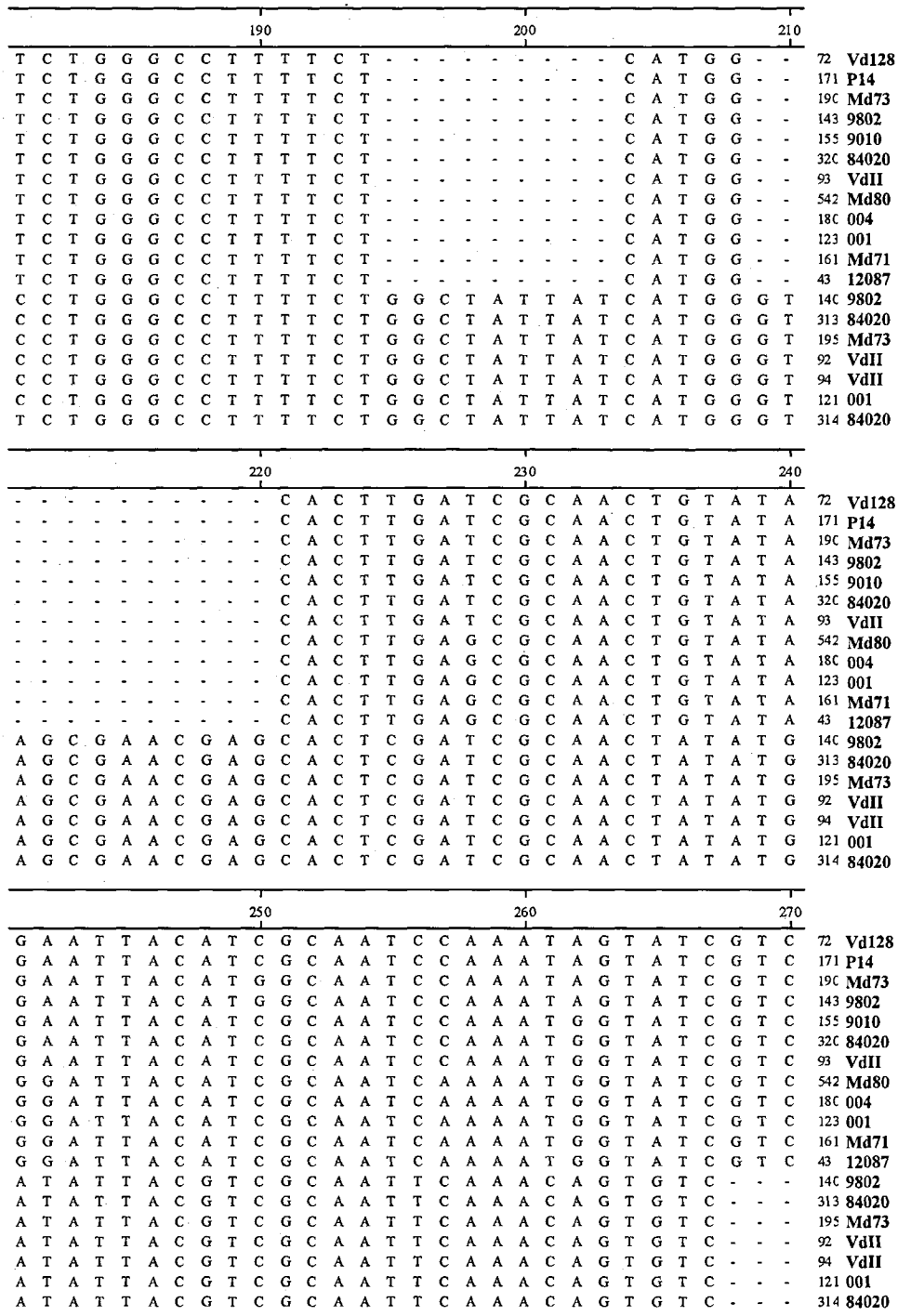

Figure 6.12. Alignment of *5S* rRNA IGR typical sequences from each clade of Figure 6.14. Numbers are clones identifiers. Letter and numbers in bold are isolate names. (See Table 6.1). Continued.

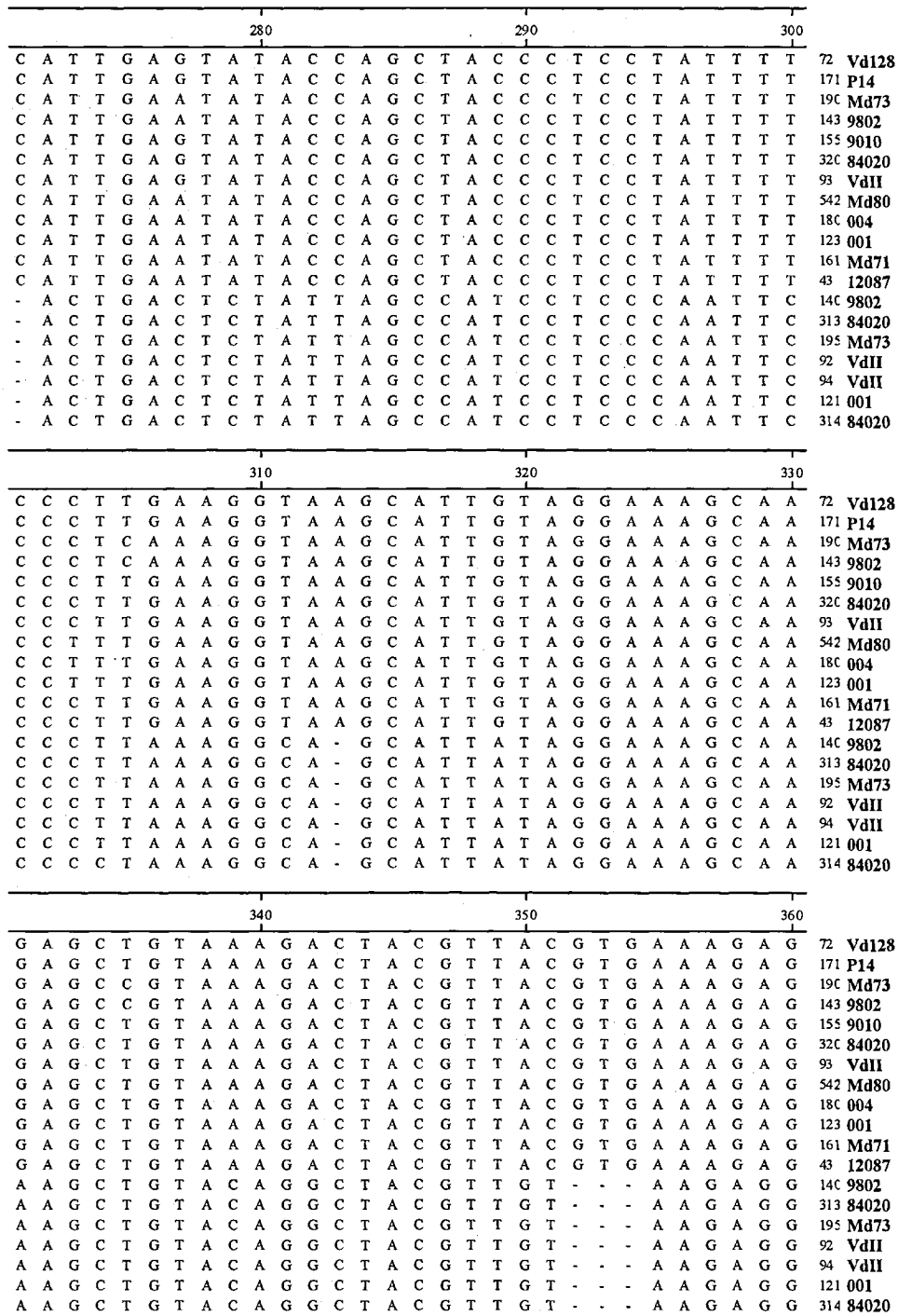

Figure 6.12. Alignment of 5*S* rRNA IGR typical sequences from each clade of Figure 6.14. Numbers are clones identifiers. Letter and numbers in bold are isolate names. (See Table 6.1). Continued.

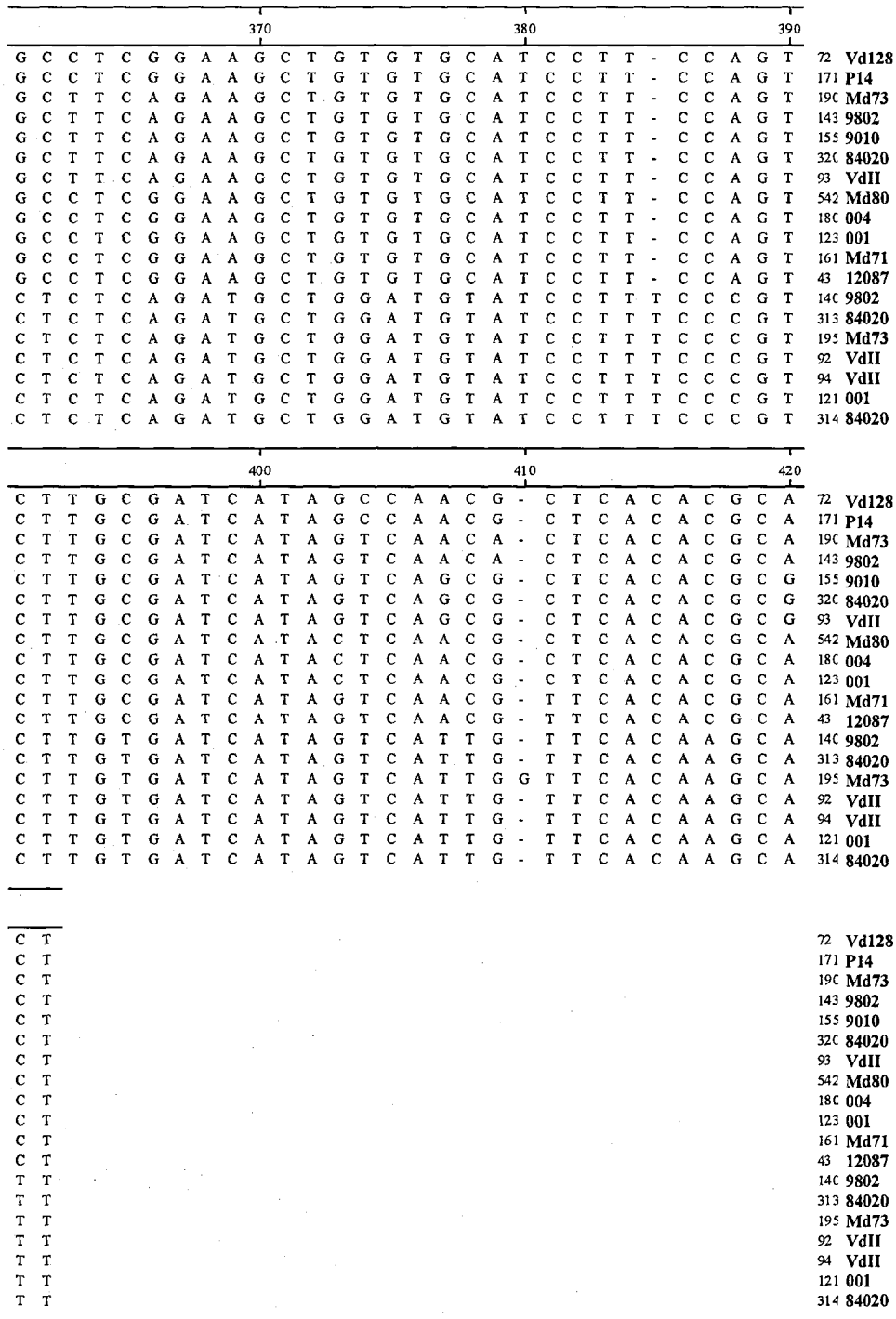

Figure 6.12. Alignment of 5*S* rRNA IGR typical sequences from each clade of Figure 6.14. Numbers are clones identifiers. Letter and numbers in bold are isolate names. (See Table 6.1).

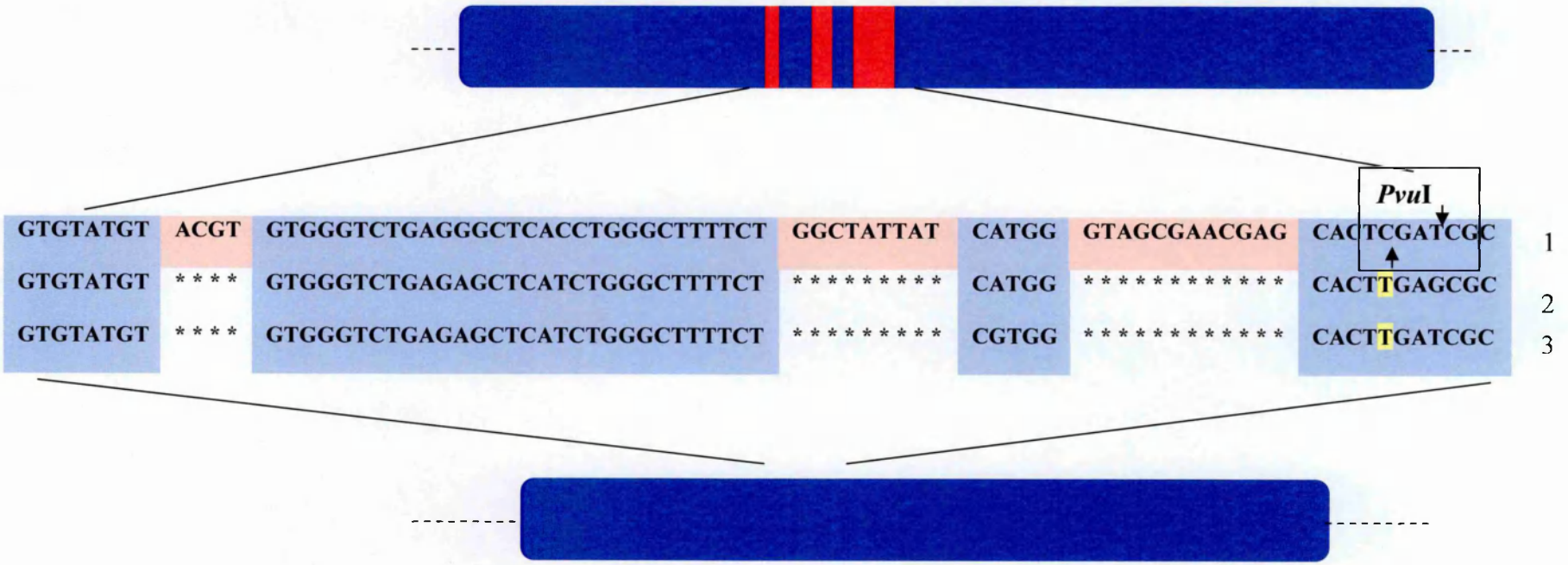

Figure 6.13. Schematic drawing of 5*S* rRNA gene IGR to show main insertion/deletions. 1: Typical long sequence; 2 & 3: typical short sequence. *Pvu*I site shown to indicate that the larger sequence would be cut by this enzyme during the Southern blotting but neither of the shorter sequences would be due to the sequence difference shown in yellow.

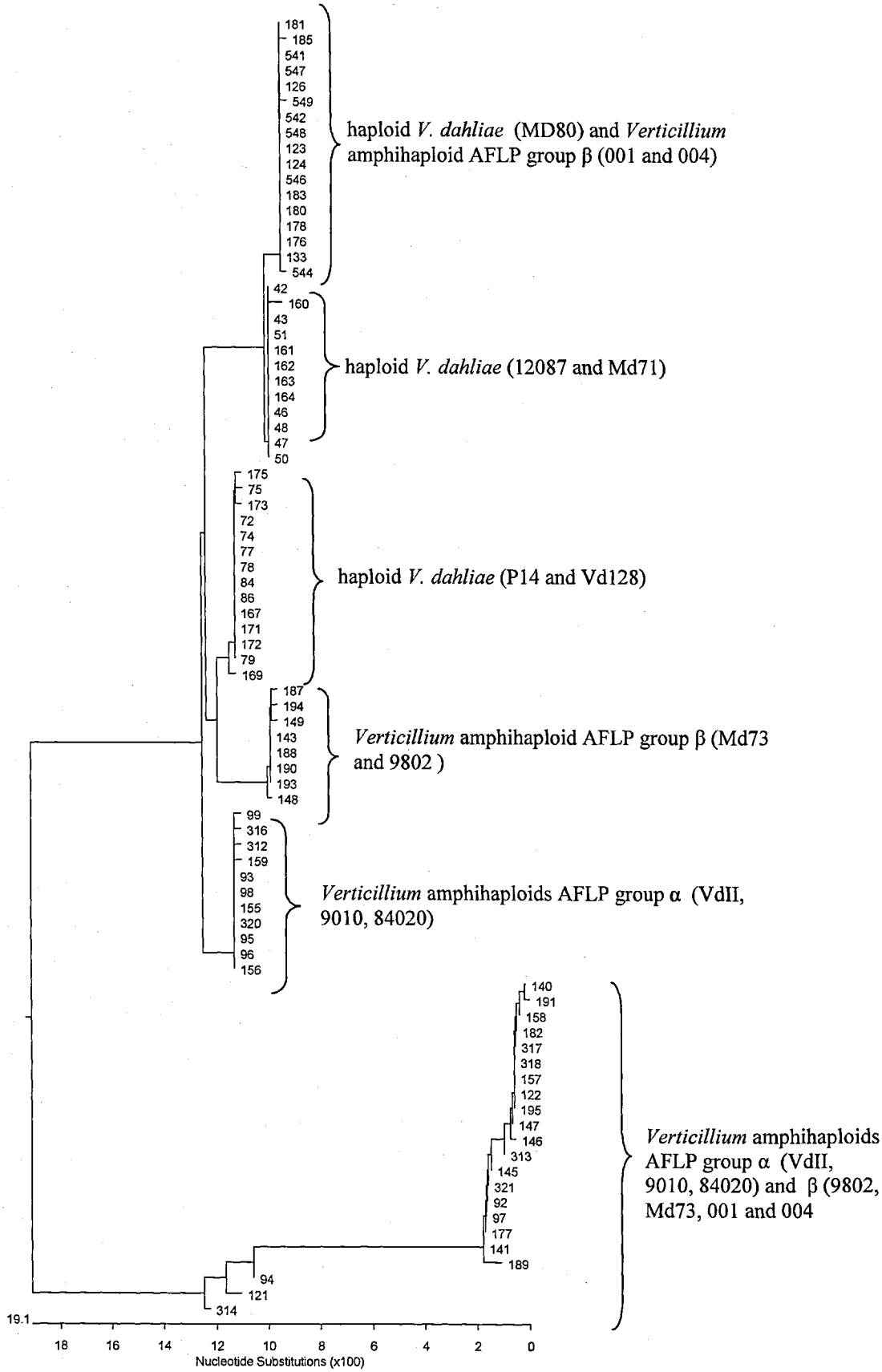

Figure 6.14. Cladogram of sequence analysis of nucleotides of 5*S* rRNA IGR amplified with primers 846/847. Numeral at the end of branches are clone identifiers. Multiple clones of each isolate were sequenced (see Table 6.1). Generated using ClustalW in MegAlign, Lasergene v 5.07 DNAStar Inc.

6.3.5 Southern analysis of 5*S* rRNA gene

Southern analysis showed that in plant pathogenic *Verticillium*, the 5*S* rRNA genes is dispersed throughout the genome (Figure 6.15 a, b). The profiles from both enzymes were scored for the presence (1) or absence of a band (0). Isolates 001 and VdII produced weak profiles when digested with *Pvu*I. Cluster analysis of the RFLP provided three main points of interest (Figure 6.16a, b, c). Firstly, the 5*S* rRNA gene patterns distinguished between haploid *V. dahliae*, *V. albo-atrum*, and *Verticillium* amphihaploids. *V. nubilum* isolate 130213 and *V. tricorpus* isolate 1988 were shown to be distinct from the main group of plant pathogens, however, *V. albo-atrum* (GpII) isolate 151, was by this measure found to be as related to *V. albo-atrum* (NL) isolates1974 and VA1, as was *V. albo-atrum* (L) isolate STR1, although all were distantly related (Figure 6.16a-c).

Secondly, for AFLP group β isolates at this locus, isolate Md73 was shown to be genetically distinct from 001, 004, and 9802. In the tree generated by *Nru*I, isolate 9802, 001 and 004 were shown to be the same, and although similar to Md73 they were distinct from it. The tree generated by *Pvu*I showed isolates from AFLP group β to be variable and similarly distinct from each other. The concatenated tree showed that 9802 was distinct group from Md73, 001 and 004 (Figure 6.16c). AFLP group α isolates always clustered together but were shown to be somewhat variable within their clade. Finally, *V. dahliae* haploid isolates formed a distinct clade with some variation within it, all isolates being distinct. (Figure 6.16c).

To better understand the relative phylogenetic position of the putative unknown parent of the amphihaploid in relation to haploid *V. dahliae*, *V. albo-atrum etc.* bands that were

common to *V. dahliae* (one of the presumed parents) were removed from the scored bands for the amphihaploid isolates and the cluster analysis repeated (Figure 6.16d). With "haploid *V. dahliae*" bands removed, *Verticillium* amphihaploid isolates formed a very distinct clade from haploid *V. dahliae*, *V. albo-atrum* and the other plant pathogenic species included in the study. Within this clade of *Verticillium* amphihaploid isolates, AFLP group α and group β isolates formed broad sub-groups distinct from each other. In addition, within the AFLP group β, isolates 001 and 004 fell together (although showed some dissimilarity between them but this may be accounted for by lack of a result for *Pvu*I), and were separate from 9802 and Md73, which were also quite divergent from each other.

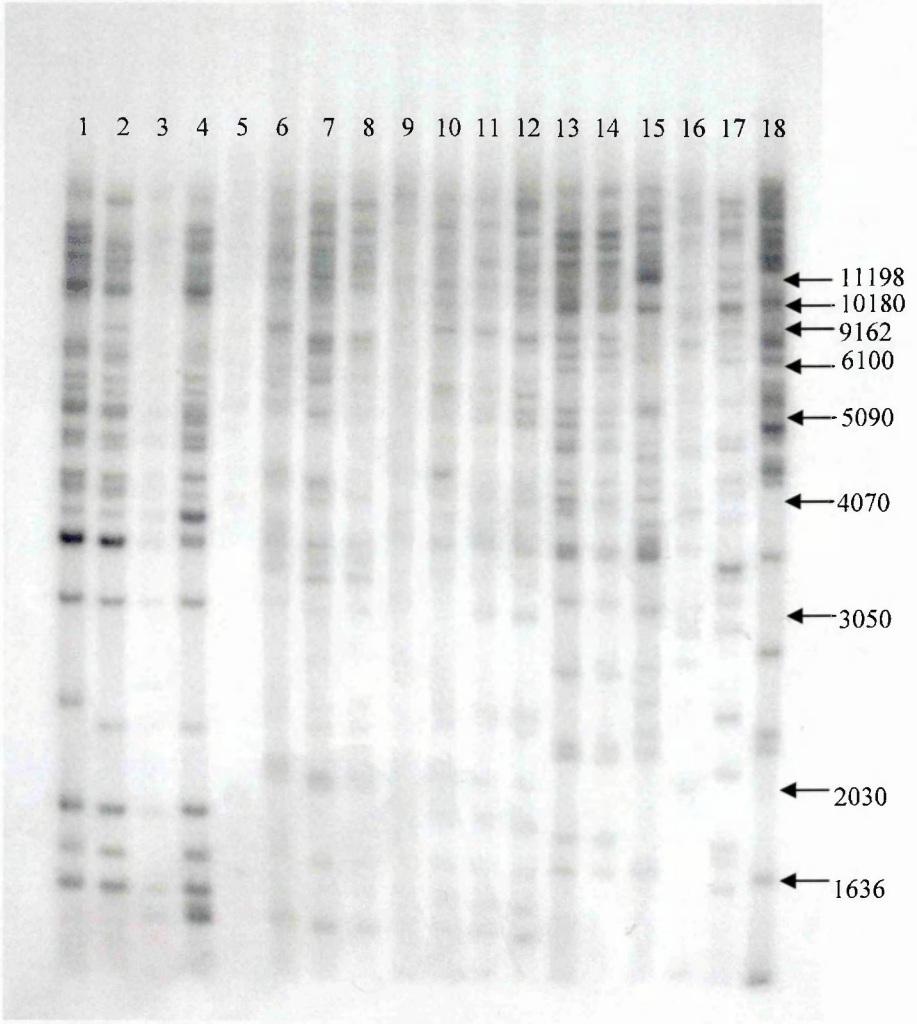

Figure 6.15a. Southern blot of genomic DNA digested with *Nru*I probed with 5*S* rRNA gene probe Lanes 1-5 haploid *V. dahliae* (1: Md71, 2: 12087, 3: P14, 4: Md80, 5: Vd128), lanes 6-8 *Verticillium* amphihaploid AFLP group α isolates (6:VdII, 7: 9010, 8: 84020), lanes 9-12 *Verticillium* amphihaploid AFLP group β isolates (9: 9802, 10: Md73, 11: 001, 12: 004), lanes 13-14 *V. albo-atrum* (NL) (13: 1974, 14: VA1), lanes 15 *V. albo*-atrum (L) (15: STR1), lane 16 *V. nubilum*: 130213, lane 17 *V. tricorpus* (17:1988), lane 18: *V. albo-atrum* (GpII) (18:151).

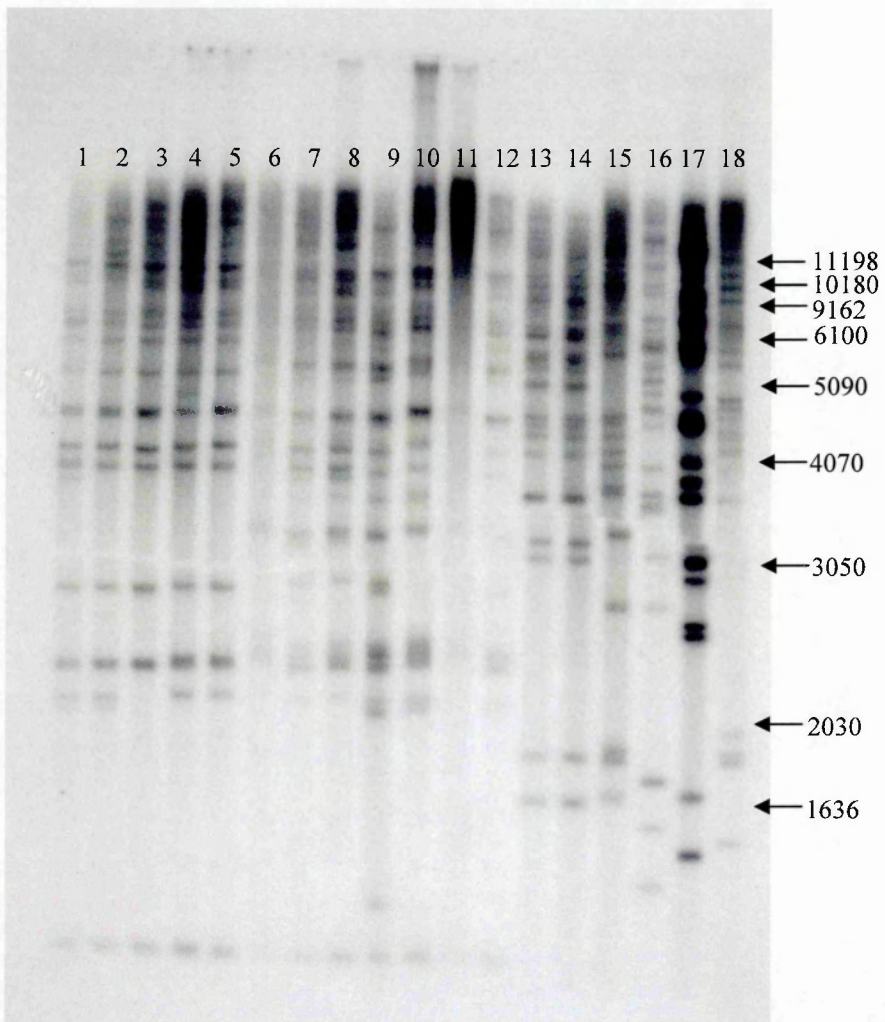

Figure 6.15b. Southern blot of genomic DNA digested with *Pvu*I probed with 5*S* rRNA gene probe Lanes 1-5 haploid *V. dahliae* (1: Md71, 2: 12087, 3: P14, 4: Md80, 5: Vd128), lanes 6-8 *Verticillium* amphihaploid AFLP group α isolates (6:VdII, 7: 9010, 8: 84020), lanes 9-12 *Verticillium* amphihaploid AFLP group β isolates (9: 9802, 10: Md73, 11: 001, 12: 004), lanes 13-14 *V. albo-atrum* (NL) (13: 1974, 14: VA1), lanes 15 *V. albo*-atrum (L) (15: STR1), lane 16 *V. nubilum*: 130213, lane 17 *V. tricorpus* (17:1988), lane 18: *V. albo-atrum* (GpII) (18:151).

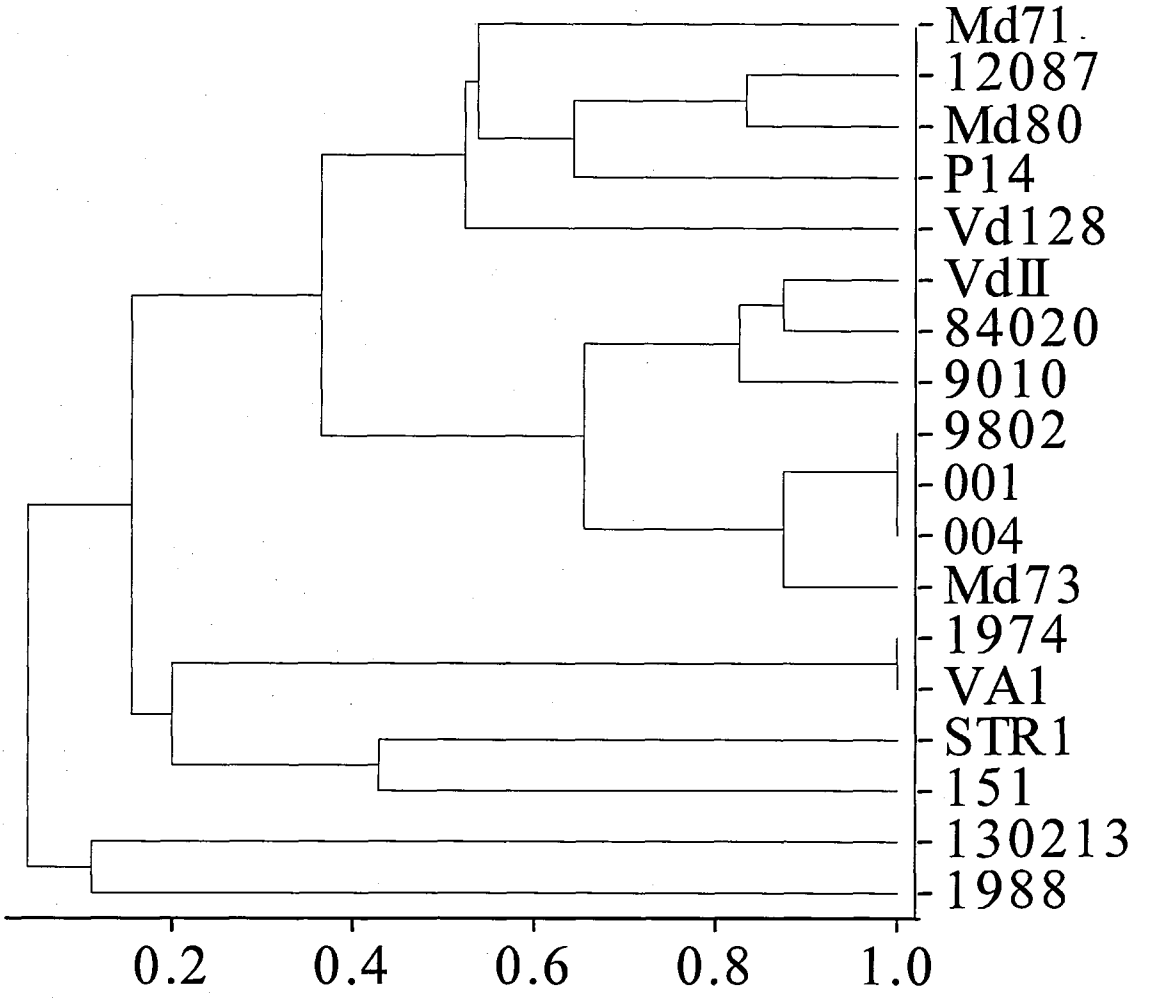

Figure 6.16a. Cladogram of *Nru*I based on 5*S* rRNA Southern blotting as seen in Figure 6.15a (only bands ≤5 kbp were scored).

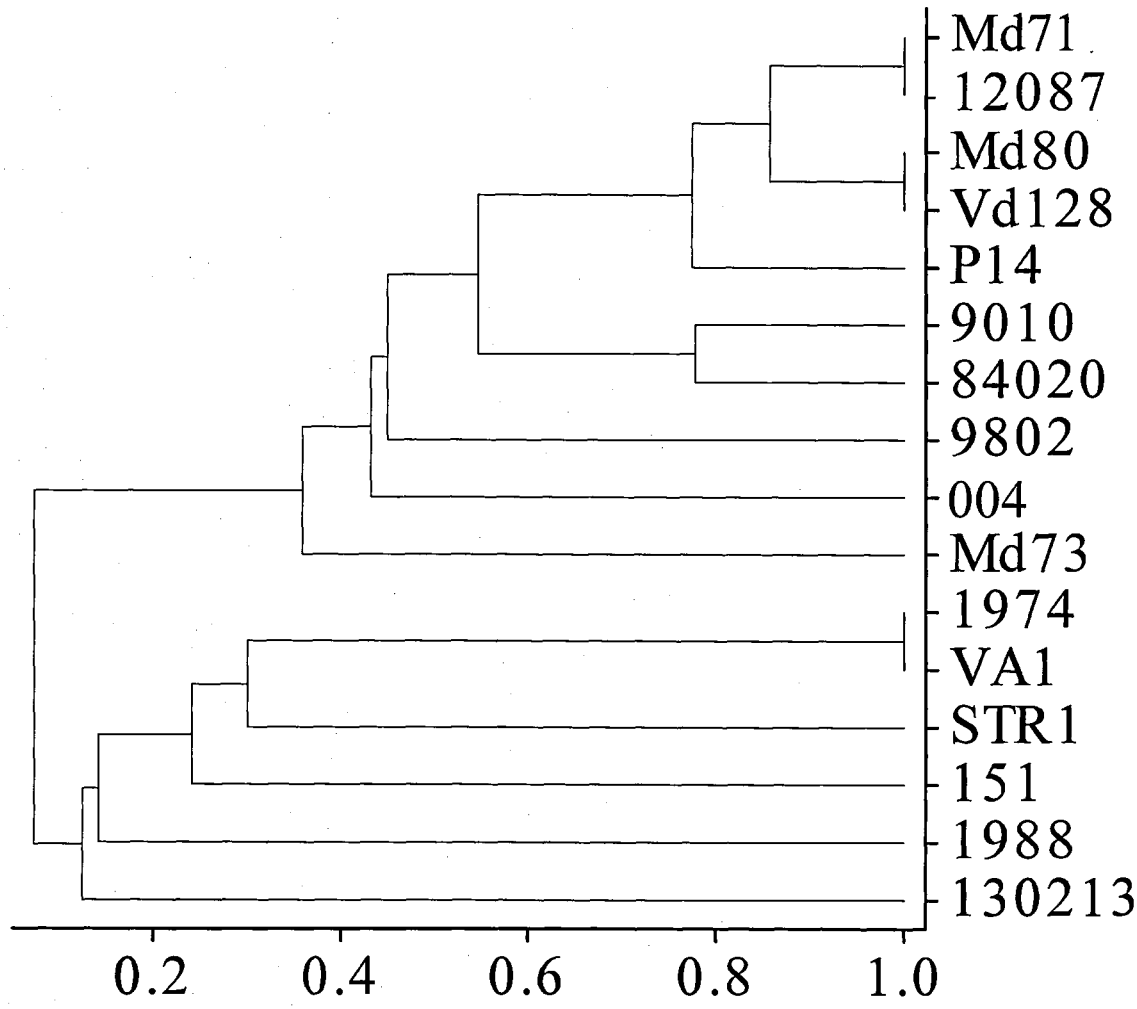

Figure 6.16b. Cladogram *PvuI* based on 5*S* rRNA Southern blotting as seen in Figure 6.15b (only bands ≤5 kbp were scored).

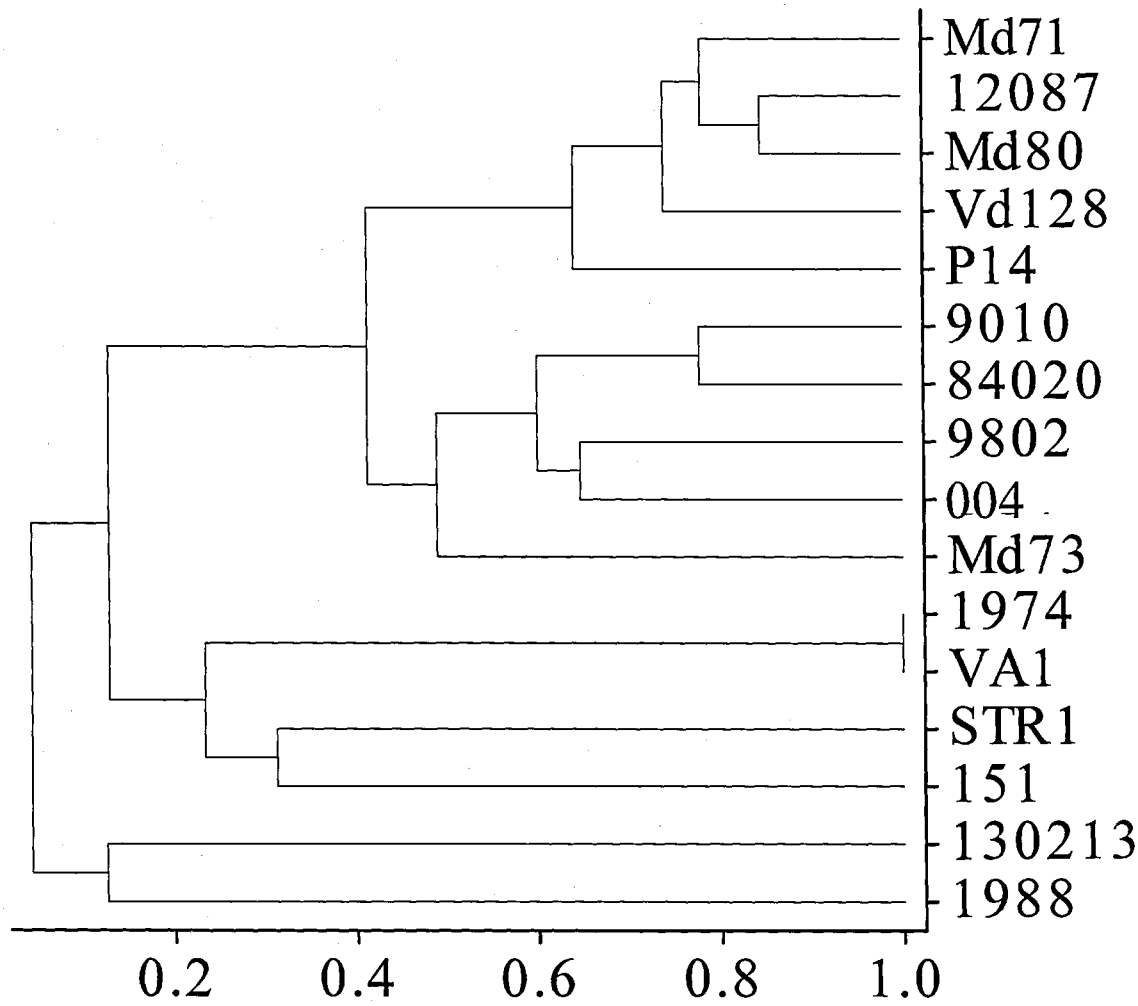

Figure 6.16c. Cladogram of data from *Nru*I and *Pvu*I combined to give a single tree based on 5*S* rRNA Southern blotting as seen in Figure 6.15a,b (only bands ≤5 kbp were scored).

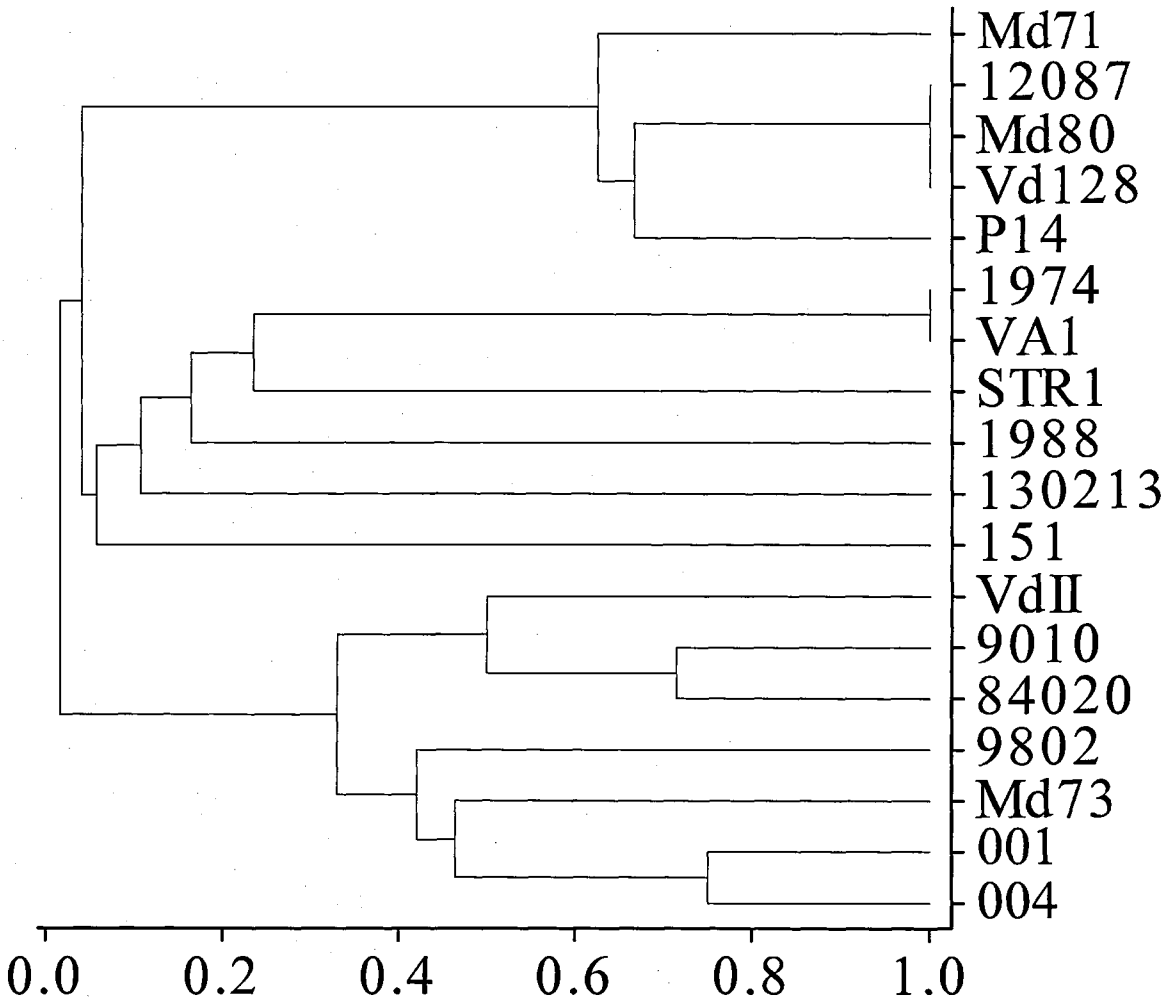

Figure 6.16d. Cladogram of data from *Nru*I and *Pvu*I combined to give a single tree based on 5*S* rRNA Southern blotting as seen in Figure 6.15a,b (only bands ≤5 kbp were scored) with *V. dahliae*-like bands removed to obtain a relative phylogenetic position of the unknown parent.

6.4 Discussion

6.4.1 Mitochondrial cytochrome B gene

These results of sequence analysis of the mitochondrial cytochrome B gene agree with results of the mitochondrial DNA RFLP analyses undertaken by Collins *et al.* (2003), that split Illinois horseradish isolates, 001 and 004, from their AFLP group β counterparts, 9802 and Md73. 001 and 004 by mtDNA RFLP are similar to haploid *V. dahliae* isolates whereas 9802 and Md73 fall with the AFLP group α isolates. Importantly though, in this study and the work of Collins *et al.* (2003) evidence is provided that *V. albo-atrum* is distinct from both haploid *V. dahliae* and *Verticillium* amphihaploids.

During the course of this work sequences of the mitochondrial cytochrome B gene from *Verticillium* amphihaploids were deposited on the NCBI GenBank database (Accession numbers : AY494994 – AY495022) and later published (Fahleson *et al.*, 2004). Results presented of the mitochondrial cytochrome B gene with other loci and AFLP, published from the same group as abstracts of the International Congress of Plant Pathology 2003 (Johansson *et al.*, 2003) led the authors to conclude that "*V. dahliae* and *V. longisporum* (amphihaploid *Verticillium*) have diverged on a species level" and that "*V. albo-atrum* has played a role in the evolution of *V. longisporum*". In the more recent publication (Fahleson *et al.*, 2004) they are somewhat more tentative and state that *V. albo-atrum* is closely related. These statements agree with previous publications, but closer inspection of the results indicates lack of scientific questioning on the part of the authors where oddities occur and thus a failure to explain them as such. The possible role of *V. albo-atrum* in the evolution of amphihaploid *Verticillium* isolates will be discussed further.

6.4.2 β-tubulin gene

Sequence analysis of the β-tubulin region amplified by primers 945/946 provides evidence that *Verticillium* amphihaploids are distinct from *V. albo-atrum* at the nucleotide level of coding sequence and the first and second intron. Sequence analysis of the amino acids for the putative protein indicates high interspecific homology, thus providing evidence that the expected gene had been amplified. However, there are some oddities that to an extent cannot be explained away through sequencing errors or mis-reading in PCR. Of course, sequencing of the mature mRNA would resolve differences seen at the amino acid level, and for further work this would be necessary; however this would not account for the majority of differences observed within the introns although it would determine the coding sequence/intron limits. This would be of particular interest with regard to the 'missing' 7aa region of the *Verticillium* β-tubulin, thought to be caused by a deleted 'G' nucleotide causing the amino acids to shift out of frame at this point, and seemingly be incorporated into the intron.

The highly divergent sequence derived from clones 205 (*Verticillium* amphihaploid AFLP group β isolate 9802) 217 and 221 (*Verticillium* amphihaploid AFLP group β isolate 001), 251 (*Verticillium* amphihaploid AFLP group β isolate Md73), 501 and 504 (*V. dahliae* isolate Md71) in all cladograms present an interesting conundrum with regard to their origin. It may suggest the involvement of a third species in hybridisation. An alternative source may be that they are divergent paralogues amplified by the same set of primers. A precedent for this is reported in studies of *Fusarium solani* (O'Donnell and Cigelnik, unpublished as cited by O'Donnell and Cigelnik, 1997; O' Donnell, 2000) where divergent paralogues are reported to be amplified by the same set of primers that would amplify one

orthologue within the *Gibberella fujikuroi* species complex. However, to some extent at this stage there is no means to prove either possible hypothesis, and so for this thesis it is more prudent to concentrate on the main body of sequences than that of these highly divergent sequences.

The objective of these studies of the β-tubulin gene was to use a functional nuclear coding gene that would present evidence of the hybrid nature of *Verticillium* amphihaploid isolates and additional evidence to their 'parental' origins. An unexpected result, was that in some *Verticillium* amphihaploid isolates there was some discordance between single clones of intron types (Tables 6.2; 6.3), and as such the inferred similarities differ between trees. This divergence between intron 1 and intron 2 sequences suggest that they may have been re-combined following an interspecific hybridisation event, and have not undergone interlocus homogenisation. These genes may have escaped this phenomenon through physical constraints within the genome, possibly a centromeric location.

A similar scenario is reported in the rRNA gene repeat ITS2 of the *G. fujikuroi* species complex (O'Donnell and Cigelnik, 1997). In these studies it is reported that discordance of ITS2 phenogram is through 'paralogous or xenologous ITS2 sequences', with both present in every isolates of the in-group species, that were designated type I and type II. Only the most frequently occurring, or major type, could be discerned through direct sequencing of the PCR products, but the less frequently occurring or minor type was detected through specific primers. This phenomenon within ITS types was studied in *Verticillium* amphihaploids, as mentioned in the introduction of this chapter, by Collins *et al.* 2003, and was not found to occur consistently in *Verticillium* amphihaploids. Although, it could be suggested in hindsight that the use of *V. albo-atrum* 'specific' ITS primers may have

introduced a significant bias away from detecting a 'minor-type' ITS sequence in the amphihaploids for reasons that shall be come apparent, but nevertheless at this stage and using this locus and sequence analysis of the intron 1 and 2, it seems that both pathotypes of *V. albo-atrum* are sufficiently distinct from *Verticillium* amphihaploids to eliminate this species as a putative 'parent'.

6.4.3 5*S* rRNA gene and IGR

Amplification of the 5*S* rRNA IGR of *Verticillium* amphihaploid isolates using primers 846 and 847 that face outward from the functional gene, provides direct molecular evidence that these isolates are hybrid. Furthermore the weak variable amplification of *V. albo-atrum* isolates with these primers, with no products of similar size to that of *V. dahliae* or the larger of the two amplicons produced by *Verticillium* amphihaploids, discounts this species as a putative 'parent' of the interspecific amphihaploid hybrid isolates, and contradicts evidence produced by other authors (Karapapa *et al.*, 1997; Karapapa and Typas, 2001; Johansson *et al.*, 2003; Fahleson *et al.*, 2004). Here evidence is given that *V. albo-atrum* did not play a role in the evolution of *Verticillium* isolates from crucifers.

Southern analysis of the 5*S* rRNA gene showed that they are dispersed throughout the genome in multiple copies, however amplification using the 'outward facing' primers 846 and 847 and sequence analysis showed that at least two genes were in close proximity to each other. Southern analysis indicates that this is not the case for the majority of 5*S* rRNA genes but it is apparent that through a theoretical hybridisation event, these two sequences,

although sharing some similarity, were brought together and did not undergo homogenisation.

As mentioned in the introduction for this chapter, the endophyte *Epichloë* Lp1 was found to have retained only a single parental 5*S* rRNA gene type (Ganley and Scott, 2002) and unlike in *V. dahliae* and *Verticillium* amphihaploids, no two genes were found to be in close proximity separated by a distinct IGR. However, as also mentioned *N. tabacum*, an allopolyploid hybrid of *N. tomentisoformis* and *N. sylvestris*, was shown to have two distinct IGRs with a length polymorphism that could be traced back to that of the parental genomes. This incongruency in genetic mechanisms across kingdoms is perhaps not unexpected, but for there to be more similarity in the perpetuation of parental 5*S* rRNA gene types between *Verticillium* amphihaploids and *N. tabacum* rather that *Verticillium* amphihaploids and *Epichloë* Lp1 does seem initially somewhat unusual but may simply indicate different chromosomal locations relative to the positions of the centromeres. It seems that *Verticillium* amphihaploids are unusual for fungal interspecific hybrids, in that these two 5*S* rRNA genes separated the IGR, not to have undergone homogenisation and produced existing paralogues and not selecting for one of either IGR sequence in successive generations. Thus from the 5*S* rRNA gene and IGR should the 'unknown' parent be found, at a molecular level it should be possible to unequivocally identify it using this marker, not withstanding the effects of gene conversion, mutation etc.

6.5 Concluding remarks

This chapter present presents evidence for three significant conclusions in research of *Verticillium* amphihaploids.

- Direct molecular evidence that *Verticillium* amphihaploid isolates are interspecific hybrids.
- There is no evidence to support that *V. albo-atrum* (as it is found today) is a 'parent' of *Verticillium* amphihaploids.
- *Verticillium* amphihaploid AFLP group β can be divided further on the basis of 5*S* rRNA IGR, mtDNA RFLP (Collins *et al.*, 2003), mtDNA cytochrome B gene. A new third group of *Verticillium* amphihaploid isolates, group γ is formed of isolates from horseradish in Illinois.

7 GENERAL DISCUSSION

7.1 Summary of results

Results from pathogenicity testing carried out in this thesis were inconsistent and as a consequence the host-specificity of these long-spored isolates to cruciferous crops remains somewhat undetermined. However, there was some degree of distinction between amphihaploids and haploid *V. dahliae* non-cruciferous isolates particularly those in AFLP group α. Whether hybridisation has conferred a selective advantage upon the amphihaploid isolates over the haploid isolates is unknown, but the reality is that for the main part, and with few exceptions, long-spored isolates in the field are solely isolated from cruciferous crops and *vice versa.*

Molecular evidence presented in this thesis clearly shows that the amphihaploid isolates are interspecific hybrids. Prior to the results presented here (Chapter 6), other authors had been able to use evidence from AFLP (Collins *et al.*, 2003) and RAPD (Karapapa *et al.*, 1997; Zeise and Von Tiedemann, 2002a) to conclude that these isolate may be hybrid in origin but were not able to give definitive proof. This thesis fulfils this requirement by providing direct molecular evidence that they are hybrids.

Analysis of amphihaploid isolates from a disease outbreak in cauliflowers from Belgium indicates that as yet there are no recent 'new' hybridisation events leading to epidemics in previously unrecognised hosts such as cauliflower in Belgium. The first report of wilt in Lucerne in Iran was confirmed to be caused by *V. albo-atrum* (L) molecularly using rRNA ITS sequence differences.

Molecular analysis of related species within the plant pathogenic *Verticillium* species complex using existing molecular markers (rRNA ITS and V-region) indicated that species such as *V. nubilum*, *V. nigrescens* and *V. theobromae* could be discounted as parents of the hybrids. From this work (Chapter 6) it is known that a *V. dahliae*-like isolate is involved in the three supposed hybridisation events represented by groups AFLP groups α and β, and the proposed third group, γ. It is also known that the other parent in each of these hybridisation events bears little similarity to any molecularly studied isolate taken from the field to this date. What is certain is that it is not like any isolate of *V. albo-atrum* known at this time, and thus the identity of the non-*V. dahliae*-like parent remains a mystery although it should be readily identified through molecular tests based on results in this thesis. The unidentified parent may be extinct.

7.2 Future Work

Should both the putative parents of the amphihaploid isolates be identified, it would be practical to set out upon the original premise of this thesis *i.e.* to generate artificial amphihaploids through protoplast fusion without inducing auxotrophic mutants in the parents. Then moving on to analyse these artificial amphihaploids (and their haploid progeny?) through genetic, molecular and pathological analyses. Results from Chapter 3 indicate that limited genetic recombination is possible between interspecific complementation of *nit* mutants, and thus indicates that pursuit of such work may be promising should the parents be identified. To this end it is helpful that molecular characterisation undertaken in this thesis has entailed that the identification of markers for the 'parents'. When this thesis began, reliable molecular markers to identify possible

hybrids were lacking and identification of artificial amphihaploids would have had to rely upon morphological characteristics (long-spores) and so-called 'novel' pathogenicity towards cruciferous crops. It is now the case that study of possible artificial amphihaploids is facilitated.

For future work it would be useful to understand the molecular mechanisms of natural interspecific hybridisation further and the processes that drive and influence such an event. This could be undertaken initially by looking for the presence of mating type genes, or genes of similar function. Additionally, this may provide evidence on the apparent intraspecies divisions created by VCG, and may resolve the seeming inconsistency of clonal isolation in asexual fungi (not withstanding theories that parasexual recombination may occur in nature) with the fact that they are molecularly highly variable, possibly associated with new disease outbreaks arising on new hosts. Analysis of mating type genes could also prove or disprove the existence of 'cryptic' sexuality in *Verticillium*, as neither a parasexual or sexual stage has been found in the field.

It is also apparent that further clarification of the species boundaries within the plant pathogenic *Verticillium* species complex (particularly the lesser studied species such as *V. nigrescens, V. nubilum* and *V. theobromae*) is required and some consideration of the taxonomy of the long-spored isolates is necessitated. The controversy regarding the erection of a new species for all long-spored isolates is well documented (Karapapa *et al.*, 1997; Collins *et al.*, 2003; Barbara and Clewes, 2003), and until such a time where the parental origins of all the amphihaploid isolates are resolved (probably as indicated through this thesis they have three distinct origins) the retention of the name *V. dahliae* proposed for all plant pathogenic species producing only microsclerotia.

7.3 'Hopeful monsters'

There are two important questions that remain unanswered, namely 'where and how did these interspecific amphihaploid isolates originate and form?' Furthermore, these questions can be either preceded or followed by the question 'what are the parents?', and it is to this last question that much of this thesis refers and has to some extent answered.

The frequency of such hybridisation events is unknown but all the evidence is that there has been three significant hybridisation events leading to disease outbreaks and are represented by the subdivision of the amphihaploids into α, β, and γ groups. It is also likely that the hybridisation events all took place in a common but yet unidentified place, as the molecular data indicates that the unknown parent seems to be common in each group of the amphihaploids (whereas the *V. dahliae*-like parent is different in the three). This unidentified locale is unlikely to be in the United Kingdom, as there has yet to be a major outbreak of *Verticillium* wilt due to long-spored isolates in this country and the threat to this country, remains as ever, through the accidental import of long-spored isolates.

The origins of short-spored *V. dahliae* isolates from crucifers are also a complex issue. That they are descendents of amphihaploid isolates is not certain. It had been assumed that they were but all the molecular evidence indicates that they are not and are variants from the main body of haploid *V. dahliae* isolates. Pathogenicity testing of these short-spored crucifer isolates was inconclusive as to any host-specificity for crucifers, and their apparent host origin may simply be misleading as to theories of where and how these isolates originated.

It should not be discounted that hybridisation between a native *V. dahliae* and the unknown species could occur in the United Kingdom through the latter isolates' introduction. We are in a time of increasing plant movement and as a consequence their associated pathogens are also moving, coupled with a changing environment which may lead to further stress on plants. Such events may allow for increased interaction between pathogens that would not necessarily meet without the influence of human activity. In 2000, Brasier summed up the situation succinctly "They may also present hybrids (even 'hopeful monsters') with greater opportunity for expression, by providing access to new or weakened hosts". It could be suggested that instead of 'why have not these unusual amphihaploid 'hopeful monsters' caused wilt in *Brassica* (oilseed rape) in the United Kingdom?' that it should be 'when will they?'

7.4 Conclusions

Long-spored isolates of *Verticillium* associated with crucifers are interspecific hybrids between a species like *V. dahliae* and a related but as yet unidentified species. From studies of this hybrid the unknown 'parent' has been characterised at a molecular level through the 5*S* rRNA gene and IGR and β-tubulin gene introns and exons. It is certain that *V. albo-atrum,* as known today, was not involved in the evolution of the amphihaploid *Verticillium* isolates. Of the amphihaploid isolates there are three groups termed α, β, and γ representing it is proposed three individual hybridisation events.

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9 APPENDIX

9.1 Publications and Manuscripts

Published

BARBARA, D.J. & CLEWES, E. (2003). Plant pathogenic *Verticillium* species: how many of them are there? *Molecular Plant Pathology*. **4.** 297-305.

GHALANDER, M., CLEWES, E., BARBARA, D.J., ZARE, R., & HEYDARI, A. (2004). *Verticillium* wilt (*Verticillium albo-atrum*) on *Medicago sativa* (alfalfa) in Iran. *Plant Pathology*.. www.bspp.org.uk/ndr/ and in press.

DEBODE, J., CLEWES, E., DE BACKER, G., & HÖFTE, M. (2005). Lignin is involved in the reduction of *Verticillium dahliae* var. *longisporum* inoculum in soil by crop residue incorporation. *Soil Biology and Biochemistry*. **37**. 301-309.

In preparation

CLEWES, E., EDWARDS, S.G. & BARBARA, D.J. Direct evidence of the hybrid nature and parental origins of *Verticillium* amphihaploids.

CLEWES, E. & BARBARA, D.J. Plant pathogenic fungal hybrids (review).

9.2 Summary table of isolates used in these studies

Isolate	Host	Origin	Purported species	Source
90-03	*Brassica oleracea* ssp. *botrytis*	California, USA	α	SK
90-10	*B. oleracea* ssp. *botrytis*	California, USA	α	SK
VdII	*B. napus* ssp. *oleifera*	Germany	α	BH
617	*B. napus* ssp. *oleifera*	France	α	HB
334	*B. napus* ssp. *oleifera*	Germany	α	HB
84020	*B. campestris* ssp. *rapifera*	Japan	α	SH
Vd292	*B. oleracea* ssp. *botrytis*	Italy	α	MC
001	*A. rusticana*	Illinois, USA	β	DE
004	*A. rusticana*	Illinois, USA	β	DE
9802	*A. rusticana*	Germany	β	BCCM
Md73	*B. napus* ssp. *oleifera*	Germany	β	HP
Hr4	*Armoracia rusticana*	Illinois, USA	*V. dahliae*[1]	WC
Hr8	*A. rusticana*	Illinois, USA	*V. dahliae*[1]	WC
Hr14	*A. rusticana*	Illinois, USA	*V. dahliae*[1]	WC
Hr24	*A. rusticana*	Illinois, USA	*V. dahliae*[1]	WC
Hr28	*A. rusticana*	Illinois, USA	*V. dahliae*[1]	WC
Wvl2	*B. oleracea* ssp. *botrytis*	Belgium	*V. dahliae*[1]	JD
K1	*B. oleracea* ssp. *botrytis*	Belgium	*V. dahliae*[1]	JD
O1	*B. oleracea* ssp. *botrytis*	Belgium	*V. dahliae*[1]	JD
P4	*B. oleracea* ssp. *botrytis*	Belgium	*V. dahliae*[1]	JD
S3	*B. oleracea* ssp. *botrytis*	Belgium	*V. dahliae*[1]	JD
Be1	*B. oleracea* ssp. *botrytis*	Holland	*V. dahliae*[1]	JD
Pf1	*B. oleracea* ssp. *botrytis*	Germany	*V. dahliae*[1]	JD
Po32nitM	*B. napus* ssp. *oleifera*	Poland	*V. dahliae*[1]	MRG
Po180nit1	*B. napus* ssp. *oleifera*	Poland	*V. dahliae*[1]	MRG
NR1-fito	*B. napus* ssp. *oleifera*	Europe	*V. dahliae*[1]	DG
NR2-fito	*B. napus* ssp. *oleifera*	Europe	*V. dahliae*[1]	DG
Md80	*B. napus* ssp. *oleifera*	Germany	*V. dahliae*[2]	HP
Vd128	*B. oleracea* ssp. *botrytis*	Germany	*V. dahliae*[2]	MC
Md71	*Matricaria chamomilla*	Germany	*V. dahliae*	HP
12080	Soil	England	*V. dahliae*	DH
12087	*Fragaria anannassa*	Germany	*V. dahliae*	DH
P14	*Lycopersicon esculentum*	Brazil	*V. dahliae*	AS
115	*Gossypium spp.*	Syria	*V. dahliae*	RR
V-017	*Cynara scolymus*	Spain	*V. dahliae*(D)	RJD
1381	*Gossypium spp.*	Spain	*V. dahliae*(D)	RJD
1771	*Gossypium spp.*	Spain	*V. dahliae*(D)	RJD
PE6902	unknown	Unknown	*V. dahliae*	HWP
PE69212A	unknown	Unknown	*V. dahliae*	HWP
PE6971	*Pisum*	Canada	*V. albo-atrum*	HWP
PE6972	*Pisum*	Canada	*V. albo-atrum*	HWP
PE6973	*Pisum*	Canada	*V. albo-atrum*	HWP
P1855	Spinach seed	Canada	*V. albo-atrum*	HWP
P1856	*Solanum tuberosum*	Canada	*V. albo-atrum*	HWP

P2934	Soil under *S. tuberosum*	Canada	*V. albo-atrum*	HWP
P2935	Soil under *S. tuberosum*	Canada	*V. albo-atrum*	HWP
VA1	*L. esculentum*	Netherlands	*V. albo-atrum*	AT
1844	*Humulus lupulus*	England	*V. albo-atrum*	WHRI
1953	*H.s lupulus*	England	*V. albo-atrum*	WHRI
1974	*H. lupulus*	England	*V. albo-atrum*	WHRI
11041	*H. lupulus*	England	*V. albo-atrum*	WHRI
Ir1	*Medicago sativa*	Markazi, Iran	*V. albo-atrum* (L)	MG
Ir2	*M. sativa*	Markazi,Iran	*V. albo-atrum* (L)	MG
Ir3	*M. sativa*	Markazi,Iran	*V. albo-atrum* (L)	MG
STR1	*M. sativa*	Canada	*V. albo-atrum* (L)	KB
STR3	*M. sativa*	Canada	*V. albo-atrum* (L)	KB
KRS1	*M. sativa*	Canada	*V. albo-atrum* (L)	KB
151	Soil	Canada	*V. albo-atrum* (II	JR
VA104	Soil under *S. tuberosum*	Prince Edward Island	*V. albo-atrum* (II)	HWP
VA175A	*S. tuberosum*	Holland	*V. albo-atrum* (II)	HWP
PE5901	unknown	Prince Edward Island	*V. albo-atrum* (II)	HWP
1988	*L. esculentum*	England	*V. tricorpus*	WHRI
Vd.LH.25	unknown	Israel	*V. tricorpus*	LT
130213	unknown	Scotland, UK	*V. nubilum**	IMI
964.95	Soil	Papua New Guinea	*V. nubilum*	CVS
8266	Soil	Germany	*V. nubilum*	BCCM
225818	unknown	unknown	*V. theobromae*	RC
29778	*Musa* sp.	unknown	*V. theobromae*	BCCM
15114	Soil	Canada	*V. nigrescens*	BCCM

Table 9.2. Isolates of plant pathogenic *Verticillium* used for these studies. Source: AS, Dr. A Soares; AT, Dr A Termorshuizen; BH, Dr B. Holtschulte; DE, Prof. D. Eastburn; DG, Dr D. Gkilpathi; DH, Dr D. Harris; HP, Dr H. Prillinger;HWP, Dr H. W. Platt; JD, Dr J. Debode; JR, Dr J Robb; KB, Dr K. Broersma; LT, Dr L. Tsror; MRG, MC, Prof. M. Cirulli; Dr M. Rataj-Guranowska; MG, Dr M. Ghalander; RC Dr R. Cooper; RJD, Prof. R. Jimenez-Diaz; RR, Prof. R Rowe; SK, Dr S. Koike; SH; Dr S Horiuchi;WC, Dr W. Chen; WHRI, Warwick HRI collection; [1] isolates of *V. dahliae* from cruciferous crops that are likely to be amphihaploid; [2] haploid isolates of *V. dahliae* from cruciferous crops as described by Collins *et al.* (2003).* supplied to us as *caveat emptor* with regard to its species identity as suppliers regarded it as divergent from the species. IMI, BCCM, BCCM™/MUCL; CVS, Centraalbureau voor Schimmelcultures.